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THE CHEMISTRY OF ANTIGENS AND ANTIBODIES

by

J. R. MARRACK

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PREFACE

THIS report by Professor J R Marrack of the London Hospital replaces an earlier one with the same title, No 194 in this Series, of which it is in effect an extensively revised edition The original report was prepared at the request of the Bacteriology Committee of the Medical Research Council, as a guide to further investigations into the chemical nature of the phenomena associated with immunity to infection, and was published by the Council in 1934 for the assistance of workers in this field Since then the demand for copies has testified both to the excellence of the report and to its utility for students of bacteriology the Council therefore invited Dr Marrack to revise it in the light of the new knowledge acquired during the past few years in this rapidly growing subject.

Medical bacteriologists are of necessity closely concerned with chemical studies which may lead to fuller knowledge of the conditions of life of pathogenic micro-organisms, or to better understanding of the responses—often highly specific—which these organisms evoke when they invade the human body Work on these lines, if progress is to be made, must obviously take account of fundamental advances in the science of chemistry, and it is therefore important that knowledge of these advances and of their bearing on the special problem should be made readily available to the investigators Dr Marrack has accordingly collected the more important results of recent experimental work upon the chemistry of antigens and antibodies, and has reviewed that subject in the light of present-day knowledge of molecular structure The Council are much indebted to him for undertaking this task in the first instance, and now for bringing the work up to date

MEDICAL RESEARCH COUNCIL,
38 Old Queen Street,
Westminster, S W 1

9th February, 1938

ADDENDA

Page	*Para*	*Line*	
89	4	5	After " proteins ", *insert* " The quantitative studies of Kabat and Heidelberger (1937) indicate that tyrosine (and possibly histidine), while important in determining the serological activity of egg albumin, have little connection with the specificity of serum albumin "
127	4	11	After " precipitin ", *insert* " Eagle (1936) showed that when the antitoxin was saturated with toxin it would still combine with precipitin "
144	4	2	After " reactions ", *insert* " According to Meyer (1929), octyl alcohol and various other surface active substances had no effect on the combination of haemolysins and bacterial agglutinins with the corresponding cells "
145	3	12	After " amino N " *insert* " The precipitates formed by Wassermann lipoid antigen with syphilitic sera contain about 6 per cent of protein (Eagle, 1935) "
186	1	2	After " (Heidelberger, 1930) ", *insert* " Heidelberger and Kabat (1937) and Eagle (1938) have more recently shown that diazo-treated pneumococcus antibody actually does combine with the specific polysaccharide "

EAGLE, H (1935) *J Immunol*, **29**, 467
EAGLE, H (1936) *Ibid*, **30**, 339
EAGLE, H (1938) *J exp Med*, **67**, 495
HEIDELBERGER, M, AND KABAT, E A (1937) *Ibid*, **65**, 885
KABAT, E A, AND HEIDELBERGER, M (1937) *Ibid*, **66**, 229
MEYER, K (1929) *Z ImmunForsch*, **62**, 338

22nd September, 1938

THE CHEMISTRY OF ANTIGENS AND ANTIBODIES

BY

J. R. MARRACK, D S O, M C, M D.

CONTENTS

INTRODUCTION

DURING recent years a great volume of experimental investigations bearing on the nature of immunity reactions has appeared In this review I have attempted not only to collect the more important results of the investigations bearing on the simpler reactions, but also to co-ordinate them in a consistent theory Throughout I have striven to present as clear a picture as is possible of the materials and processes involved Many aspects of this presentation may be the subject of controversy , some may prove wholly wrong The value of a theory, however, does not lie in its immunity to criticism, but in its suitability for experimental test and its applicability to practice For example, the statement that any particular reaction is a complex interaction of colloids and salts may be above criticism it is also above test or application it leaves us where we were If the result achieved here is unsatisfactory, the fault does not lie with the line of approach

My thanks are due to the Bacteriology Committee of the Medical Research Council for their generous advice and assistance

CHAPTER I

PHYSICO-CHEMICAL CONSIDERATIONS

In this chapter a short and necessarily superficial account is attempted of certain aspects of physical chemistry related to the materials and processes involved in immunity reactions Particular attention has been paid to recent developments of knowledge of the shapes and sizes of molecules and of the application of this knowledge to proteins, on account of the particular importance of these developments in connexion with immunological specificity

One outstanding difficulty in immunology has been to account for the specific combination of an indefinite variety of antigens with the corresponding antibodies, in the absence of any evidence that the majority of antigens contained any specially reactive groups The conception that specificity may be determined, not only by the presence of active groups or atoms, but also by their spatial distribution, affords a solution of this difficulty , for on a large molecule a very large number of arrangements is possible But, as long as structural formulae were regarded merely as a method of representing chemical properties, such a conception remained purely hypothetical In 1917 Dean was justified in speaking of the familiar ' lock and key ' simile as ' merely a diagrammatic representation of the supposed facts ' But, with the advances of recent years, spatial distribution and its effects have ceased to be a hypothesis invented to explain the phenomena of specificity and have become a deduction from other sciences which calls for application to immunology.

It is commonly, though not universally, held that the actual combination of antigen and antibody is an instance of adsorption , that is, of a combination of molecules not due to primary valency It is therefore necessary to include some account of the properties of the inter-molecular forces (secondary valencies) which give rise to such combinations Also of the effects which these forces have on the orientation and configuration of molecules, since these effects may play an important part in the further stages of immunity reactions

A Shapes and Sizes of Molecules

The distances between the centres of atoms, found by the X-ray analysis of crystals have shown that atoms have definite radii, little affected by the nature of the compound in which the atoms are involved The radii of the atoms of chief importance in organic chemistry are shown in Table I Evidence from various sources also indicates that the angles between the valency bonds of the atoms are also constant The valency bonds of carbon (Fig I A) can be regarded as directed towards the centre of the carbon atom from the four corners of a regular tetrahedron whose centre coincides with that of the carbon atom , the angle between any pair of bonds being 109° 28′ Those of nitrogen (Fig I B) are directed towards the centre of the atom from three such corners, and those of oxygen (Fig I C) converge at an angle of approximately 109°. When two atoms are joined by a

TABLE I

Radii of atoms in Angstrom units ($\mathring{A} = 10^{-8}$ *cm*)

C (diamond structure)	0 77	Br	1 1
C (graphite ,,)	0 72	I	1 3
N	0 70	H	0 4
O	0 66	P	1 1
S	1 04	As	1 2
Cl	1 0	Sb	1 4

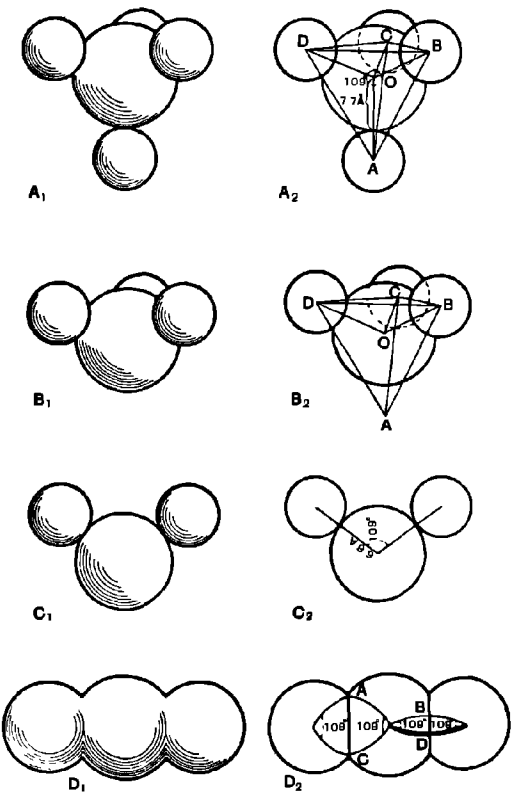


FIG I Molecular models (A) CH_4, the centres of the hydrogen atoms are situated at the corners of a regular tetrahedron *ABCD* (B) NH_3 (C) H_2O (D) CO_2 The light bond on the right is behind the plane of the paper, which corresponds to the plane *OAC* in (A) The heavy bond is in front of the plane of the paper.

double bond, the line joining their centres bisects the angle between the valency bonds of each atom (Fig I D)

From these considerations it must be inferred that organic molecules have definite shapes and sizes in agreement with their three-dimensional structural formulae This is in agreement with what is actually found both by X-ray study of crystals and the measurements of the areas of surface films For example, the molecules in a crystal

of a fatty acid such as palmitic acid have the shape and arrangement shown in Fig II The centres of the carbon atoms lie in one plane (here the plane of the paper) and are actually arranged in a zigzag chain, the distance between one carbon atom and the next but one is 2·54 Å

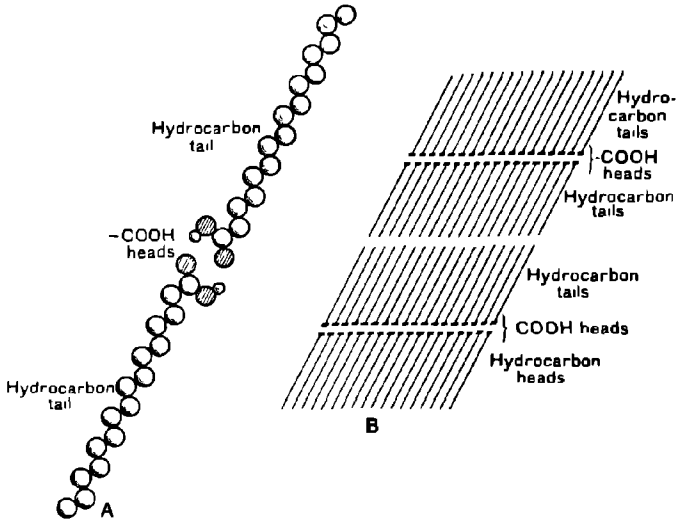
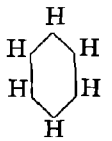
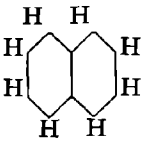


FIG II (A) Two palmitic acid molecules in two layers of a crystal, the hydrogens of the tails are omitted (B) Diagram of two double layers of a crystal of a fatty acid

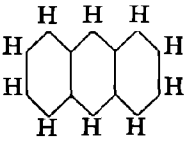
The carbon atoms of benzene actually form a ring, the lattice measurements of crystals of benzene,



naphthalene,



and anthracene,



show that the width of the molecule increases by approximately 2 5 Å with the addition of an extra ring, in agreement with the measurements given (Fig III)

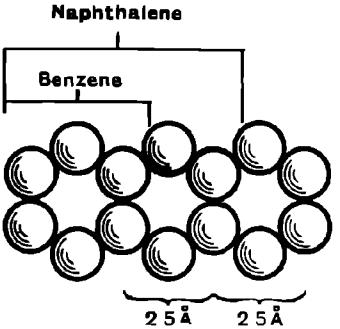


FIG. III Anthracene (H atoms omitted)

Not only have the arrangements of atoms and sizes of molecules of known substances been found to conform with their structural formulae, but X-ray measurements have also given considerable information about compounds whose structure was uncertain, as for example, cellulose and silk [1]

The shapes and sizes which can, therefore, be attributed to organic molecules must play a very important part in determining the properties of large and complicated molecules Even relatively small molecules give examples of these influences For example, maleic acid loses H_2O on heating from the contiguous —COOH groups, forming an anhydride , while fumaric acid, in which the —COOH groups are separated, does not (Fig IV)

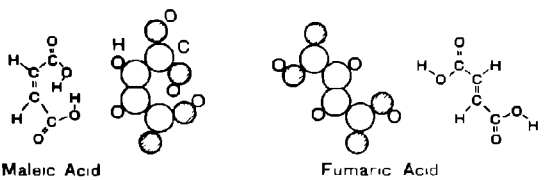
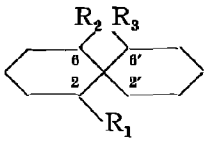


FIG IV Maleic and fumaric acids, showing the differences in the distances between the —COOH groups

[1] It may be pointed out that these conclusions about the size and shape of molecules are not in the same category as hypotheses of the structure of the atom, such as that of Bohr The latter may be described as attempts to employ the familiar concepts of matter below the range at which they are applicable Such an hypothesis can only give an incomplete description, which may be useful, as in the electronic theory of valency But when dealing with dimensions above those of atomic structure the ordinary ideas of matter can be used, and atoms and molecules can be thought of as occupying definite fractions of space, which cannot be occupied at the same time by other atoms or molecules It may be more correct to say that when two atoms approach within a certain distance the repulsive forces begin to increase very rapidly, but the same description would equally be more correct for the collision of macroscopic objects Although the knowledge of the dimensions of atoms and shapes of molecules is mainly based on X-ray analysis of crystals, it is confirmed by so many independent lines of investigation that it may be applied to chemistry generally without hesitation

Although many instances of steric hindrance are due to other causes, some are undoubtedly due to the actual bulk of parts of molecules A striking instance is that of the diphenyls (Richter, 1932)



If three of the hydrogens attached to the 2, 2', 6, 6' carbons are substituted by other atoms or radicles such as CH_3, the two rings, as shown by the optical activity of the molecules, are unable to lie in the same plane , the substituent radicles (in the example R_2 and R_3) prevent this by getting in each other's way

When dealing with molecules containing some thousands of carbon atoms and 40 Å or more across, far greater importance must be ascribed to size and shape

B Intermolecular (Polar) Forces

The importance of intermolecular forces, which hold the individual molecules of solids and liquids together, is obvious But, since the forces acting between small molecules are negligible compared with those binding the constituent atoms together, they are generally neglected in ordinary chemistry They may be considered as originating from the unequal distribution of electric charges in the molecule , for example, the hydrogen atoms of water are positively charged and the oxygen atom negatively charged, although the molecule as a whole is neutral.

1 *Electronic Theory of Valency*

Using the Bohr atom model, the differences in the chemical properties of atoms appear to be mainly due to their outer shells of electrons For in such a series as the horizontal row of the periodic table Ne, Na, Mg, Al, Si, P, S, Cl, the atoms differ from the first, neon, only in the outer shell, which increases from one electron in sodium to seven in chlorine These electrons are therefore supposed to take part in chemical reactions, and are called the valency electrons

When two atoms combine a redistribution of their electrons takes place, such that the number of electrons in the outer shell of each atom is reduced or increased to eight,[1] the number in the outer shell of the inert gases neon, argon, krypton, and xenon The system is most stable with eight electrons in the outer shell , hence the chemical inactivity of the inert gases

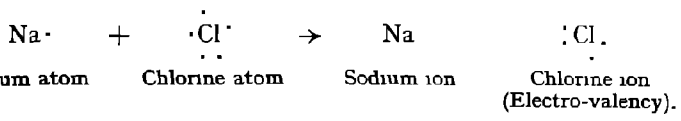
This partition of electrons can be effected in two ways

(1) One atom may give up one or more electrons to another Two oppositely charged ions are produced which are held together only by the attraction between opposite charges (electro-valency).

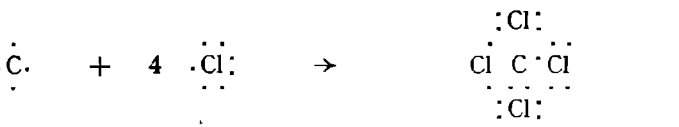
---

[1] In the case of lithium, beryllium, and boron the reduction, and in the case of hydrogen, the increase is to two electrons, the number in the only shell of the inert gas helium

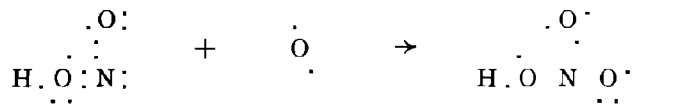
(2) Two electrons may be shared between the two atoms forming a single bond These two shared electrons may be supplied one by one atom and another by the other (co-valent bond), or both may be supplied by one atom (co-ordinated bond) In a double bond four electrons, and in a treble bond six electrons are shared It is convenient to represent the valency electrons by dots In the diagram the third oxygen of nitric acid is attached by a co-ordinated bond, since both the shared electrons are supplied by the nitrogen atom



Sodium atom Chlorine atom Sodium ion Chlorine ion (Electro-valency).



Carbon atom Chlorine atom Carbon tetrachloride (Co-valency)



Nitrous acid Oxygen atom Nitric acid (Co-ordination compound)

The electrons are not, however, stationary , the shared electrons of co-valent or co-ordinated bonds rotate round the positive nuclei of each atom in a figure of eight

The electrons in a co-valent link are not necessarily shared equally between the two atoms The loop of the figure of eight round centre of one of the atoms (A) may be larger than that round that of the other (B) , in this case the electrons will spend more time in the orbit round the nucleus of atom (A), and (A) will therefore be relatively negatively charged, and, conversely, (B) will be positively charged In a co-ordinated link the atom which supplies the pair of electrons loses them from its system for part of the time, without a corresponding gain as in a co-valent link , this atom is therefore positively charged The other atom in the co-ordinate link gains two electrons without a compensating loss, and is therefore negatively charged

The molecules formed may be, on the whole, neutral, but their constituent atoms may be positively or negatively charged and attract oppositely charged atoms of other molecules According to the theory, as here outlined, there is a difference of kind between the combination of two atoms in a molecule (in which case electrons rotate round the nuclei of both atoms) and the attraction between the atoms of different molecules (in which case the electrons are only attracted by the positive nucleus of another atom) It is possible, however, that the difference is only one of degree Nevertheless this distinction remains , combinations by co-valent or co-ordinated

links must obey laws of multiple proportions, whereas this is not the case with molecular attractions However, when molecules contain numerous combining groups of varying strength it is difficult to apply this criterion

The intensity of the fields of these intermolecular forces falls off rapidly with distance; they are therefore effective only between molecules in close proximity The strength of the fields associated with different groups of atoms varies greatly, but is approximately constant for any particular group When a liquid is heated the increased heat vibrations tend to shake the molecules apart, while the intermolecular forces tend to hold them together, the amount of heat energy that has to be supplied in order to separate the molecules of a liquid, to form a gas, is therefore a measure of these intermolecular forces The values given in Table IIA for the energy required to overcome the attraction between molecules, due to the various groups shown, are calculated from the heat of evaporation The effect of these groups in a molecule is usually additive Groups such as  which give rise to strong intermolecular forces will be spoken of as polar groups, in contrast with non-polar groups such as $-CH_2-$ and $-CH_3$, and the intermolecular forces as polar forces

With increase of the size of an organic molecule, the polar forces are increased with the addition of each group, while the energy of heat vibration per molecule does not increase Substances consisting of large molecules are therefore more rigid and have higher boiling-points than those composed of small molecules, as may be illustrated by a homologous series such as the paraffins With sufficiently large molecules, the molecules themselves are decomposed before the solid melts, that is, the energy of heat vibration breaks down the bonds between the constituent atoms before it disrupts the polar forces holding the molecules in fixed position relative one to another

Large molecules may be closely packed with their polar groups in opposition, their attraction for water, on which their solubility depends, will then be reduced in the same way as the ortho-benzene derivatives (p 20) Such close packing may result from a specially suitable shape, for example, long thread-like molecules, or from a specially strong polar attraction between certain sites on the molecules

Owing to the short range of polar forces, the spatial arrangement of polar groups on molecules has an important effect on the strength with which two are held together by these forces If two molecules have polar groups A B and A′B′, both these groups cannot be effective in holding the molecules together if in the one they are separated only by a few Å and in the other by some 10 Å, unless the molecules can be distorted As the number of polar groups and the molecular surface increases, an appropriate spatial distribution of the groups will play a greater and greater part in determining the firmness with which molecules are held together

TABLE IIA

Energy required to separate molecules against attraction due to organic groups, in calories per molecule (Meyer and Mark, 1930)

$-CH_3$	1,780	$-NH_2$	3,530
$-CH_2-$	990	$-Cl$	3,400
$=CH-$	990	$-Br$	4,300
$-O-$	1,630	$-I$	5,040
$-OH$	7,250	$-NO_2$	7,200
$=CO$	4,270		
$-C(=O)H$	4,700	$-C(=O)NH_2$	13,200
$-C(=O)OH$	8,970		
$-C(=O)CH_3$	5,600	$-C(=O)NH-$	10,600
$-C(=O)C_2H_5-$	6,230		

TABLE IIB

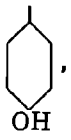
Relative strength of polar force of organic groups, in order of decreasing strength

	$-C(=O)NH_2$	$-C(=O)OH$	$-CH_2OH$	$-C(=O)H$	$-CH_2NH_2$	$-CH_3$
Example	Propionamide	Propionic acid	Propyl alcohol	Propyl aldehyde	Propyl amine	Propane
	$C_2H_5-C(=O)NH_2$	$C_2H_5-C(=O)OH$	$C_2H_5-CH_2OH$	$C_2H_5-C(=O)H$	$C_2H_5CH_2NH_2$	$C_2H_5CH_3$
M P	75° C	−36° C				
B P	decomposes	140 7° C	97 4° C	68 7° C	49° C	−37° C
Solubility in water	v sol	miscible in all proportions	sol	sol	sol	insol

2 *Orientation of Molecules by Polar Forces*

Films formed on the surface of water afford a relatively simple example of the orientation of molecules under the influence of polar forces The oxygen of H_2O is negatively charged and the hydrogen positively charged A polar group like the $-COOH$ of a fatty acid such as palmitic acid is therefore attracted by the water molecules, while the non-polar hydrocarbon tail is attracted less strongly (Fig V) If there is room, the fatty acid molecule will lie on the surface of water, but if there is insufficient room for this arrangement the $-COOH$ will be in the water surface and the hydrocarbon chains will

stick up in the air (a ' condensed ' film) The distance between the chains are then the same as in a crystal ; the film, in fact, is a crystal one molecule thick. Molecules of aromatic compounds with



,

, or

as end groups

also form mono-molecular films on water , in the condensed film the

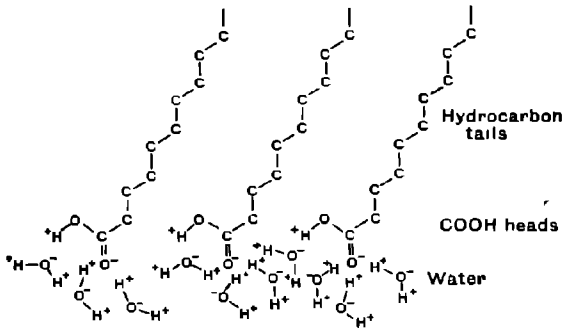


FIG V Condensed film of fatty acid on water (hydrogens of tails omitted).

ring is upright and the area occupied per molecule is approximately the same as the effective cross-section of a benzene molecule in a crystal

It is a general rule that molecules with polar and non-polar parts form mono-molecular films , when such molecules are closely packed in the film the polar part is in the water surface and the non-polar part is turned away from the water (Fig VI B) The result is the same

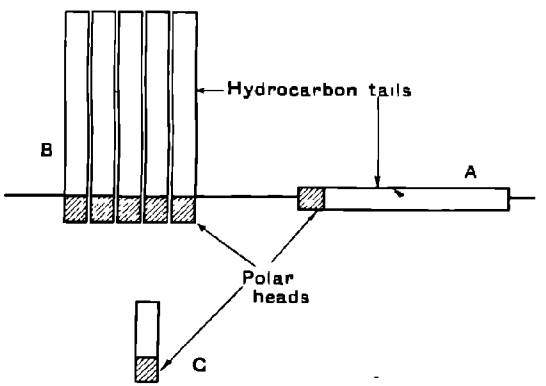


FIG VI Distribution of molecules with polar and non-polar parts in and on the surface of water (A) Position taken up on the surface when the molecules are not closely packed (B) Position when molecules are closely packed on the surface

whether the surface is one between the water and air or one between water and a non-polar liquid such as benzene or paraffin.

If, on the other hand, the molecules of a substance contain no polar groups, it will not form a film on water, because no parts of the molecules are attracted to the water surface

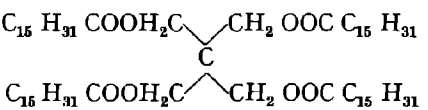
These conclusions are sufficiently well established for it to be possible to employ the study of surface films as a means of investigating the structure of unknown substances, for example, oestrin (Adam *et al*, 1932)

In the case of simple molecules such as fatty acids, the orientation in crystals is determined by the same considerations as in a film on water The arrangement produced is similar, the polar — COOH heads of one layer of molecules is turned towards those of the next layer instead of towards the water (Fig II) Molecules in which the arrangement of polar distribution forces is less simple are also oriented in relation to one another in definite positions determined by these forces and by considerations of size and shape

3 *Distortion of Molecules by Polar Forces*

Owing to the possibility of rotation round a single bond great changes may occur in the shape of organic molecules The hydrocarbon tail of a fatty acid is straight in a crystal, but may be bent when exposed to unsymmetrical fields of force and is possibly curled in a helix when the molecule is in solution

Molecules may be considerably distorted under the influence of polar forces In films this distortion may be necessary in order to bring the maximum number of polar groups into the water surface If, for example, pentaerythritol tetrapalmitate



retained its symmetrical structure in a film the polar groups could not get near the surface of the water. It forms a condensed film, however, with all four hydrocarbon chains vertical (Adam, 1928)

4 *Solution*

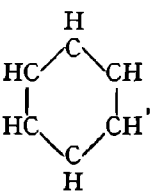
If the molecules of a substance contain polar groups, and the non-polar part is not too great, the attraction of the water molecules for the polar groups will be sufficiently great to draw the molecules from the surface into the bulk of the water, that is, into solution (Fig VI c). If the polar groups are weakened, or the non-polar part increased, the attraction of the water molecules for one another becomes stronger than their attraction for the foreign molecules and these are squeezed out of the bulk of the fluid The solution of organic molecules therefore depends on the relation between the number and strength of the polar groups and the size of the non-polar part of the molecule This is illustrated by the progressive insolubility of fatty acids with

increasing length of the hydrocarbon chain, and of compounds with the same number of carbon atoms attached to groups of diminishing polarity (see Table II B)

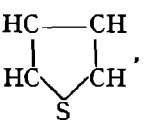
Water molecules are more or less firmly bound to the polar groups and move to some degree with the solute molecule and increase its apparent bulk The solute molecule is said to be ' hydrated ' The water of hydration is less available for the solution of other molecules, and the hydration can be detected and its extent measured by the effect on the solubility of inert gases

5 *Specificity of Binding by Polar Forces*

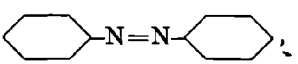
The possibility of great specificity in the binding together of molecules by polar forces is illustrated by the selection of the molecules built into crystals Such molecules have to conform to specifications with regard to their dimensions and the distribution of polar forces As a result crystals usually consist of only one species of molecule ; a fact which has its practical application in the use of crystallization for purification However, foreign molecules may be built in to form mix-crystals, actually replacing the primary molecules in the crystal lattice, if they do not deviate from the primary molecules too far in shape and size, and in the distribution and strength of polar fields Such molecules may differ considerably in constituent elements , for example, benzene



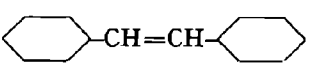
and thiophene



and azo benzene



and stilbene

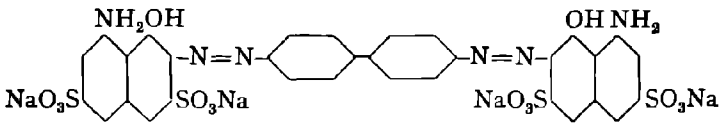


form mix-crystals

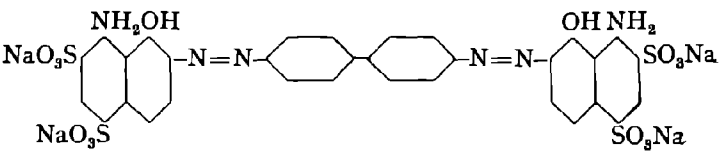
Less strict conformity is required in order that the molecules of a substance may be adsorbed on the surface of a crystal For in this case only one aspect of the adsorbed molecule need conform to the distribution of polar forces on the surface of the crystal Two types of molecules may be adsorbed in this way First those which resemble the molecules of the crystal to some degree, but not sufficiently for it to be possible that they should be built into the crystal lattice ; these

take the place of the primary molecules on the surface And, second, those which do not resemble the primary molecules but present a distribution of polar forces which fits that of the crystal surface ; such molecules do not have the same distribution on the surface of the crystal as would a further layer of the primary molecules. In either case the adsorbed substances inhibit the growth of the crystal faces on which they are adsorbed.

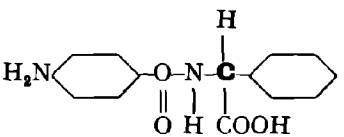
A remarkable specificity may be found in the adsorption of the second type Thus methylene blue is adsorbed by diamond and not by graphite, succinic acid by graphite and not by diamond (Nellenstein, quoted by Garner, 1926) The two adsorbing surfaces differ only in the spacing of the carbon atoms The dye



is adsorbed on the cube faces of potash alum , whereas the dye



which differs only in spatial arrangement is not adsorbed (France, 1930) Differences may even be found in the degree to which optical isomers are adsorbed Ingersoll and Adams (1922) found that wool adsorbed the laevo- and dextro-rotatory forms of the dye formed by coupling diazotized phenyl-(*p*-amino-benzoyl-amino)-acetic acid



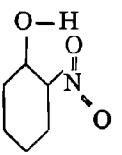
to dimethyl-aniline, unequally. The two dyes differ only in the arrangement of the groups round the C atom in heavy print Differences of adsorption in these cases cannot be attributed to differences of charge of the whole molecule, but only to differences of distribution of the polar fields of the molecules

Association between molecules, as shown chiefly by the penetration of a surface layer of one species of molecules, A, by molecules of another species, B, has been studied by Schulman and Rideal (1937) Strong association takes place only if both polar heads and non-polar tails of B are suited to those of A That between the polar groups of sodium cetyl sulphate and the polar groups of other molecules decreases in the order, sterol OH > long chain OH > phenol OH > long chain ester > sterol ester. The non-polar part of sodium cetyl sulphate is more strongly attracted

to saturated long chains and unsaturated condensed ring systems (such as that of cholesterol) than to unsaturated long chains with a double bond in the middle and saturated condensed ring systems. This association is, therefore, specific to a considerable degree.

The adsorption, even of simple molecules such as hydrogen and ethylene on apparently uniform surfaces such as those of copper and nickel, occurs mainly at suitable patches on the surface (Glasstone, 1931) It is with such adsorption, rather than the formation of films on uniform surfaces, that we shall have to deal The little work that has been done in specific adsorption has almost all been in connexion with surfaces Specific adsorption, however, can also occur between isolated molecules, and will be determined by the same factors , it will become progressively more important with the increasing size of the molecules concerned, because the firmness of union by polar forces and the possibility of a varied mosaic of polar fields increase with increasing molecular size

If the adaptation of one rigid[1] molecule to another in solution in water involves the distortion of one or both, adsorption is not likely to occur, because their polar fields can be satisfied by the free water molecules without the necessity of distortion On the other hand, if two suitable polar groups are held in proximity, their polar fields may neutralize each other and lose the attraction for water on which the solubility of the molecule depends Thus *ortho*-benzene derivatives such as o-nitro-phenol (Sidgwick and Callow, 1924)



are less soluble than the corresponding *meta*- and *para*-derivatives in which the polar groups are more widely separated [2] The mutual neutralization of the polar groups is also shown by a greater solubility in the non-polar solvent benzene, and lower melting-point (i e the molecules in crystals of these substances attract each other less strongly)

6 *Adsorption Isotherms.*

As adsorption that is, the binding of molecules by polar forces, may play an essential part in the combination of antibody and antigen, it is necessary to consider the quantitative aspects of this combination more fully

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[1] The rigidity of a molecule is mainly determined by the formation of rings, which may be joined either by chemical links or by attraction between polar groups

[2] In this case it is possible that a co-ordinated link is formed between an —O of the $-NO_2$ and the H of the —OH In any case the polar groups are neutralized as a result of proximity.

In the kinetic derivation of the law of mass action no assumption is made as to the mode of union of the molecules that combine, except that this union is reversible It should therefore be applicable when molecules are joined by polar forces, as, for example, when molecules such as benzoic acid, dissolved in water, aggregate Even when a combination on the surface of one phase with molecules moving in another phase is involved, an equation similar to that derived from the law of mass action may be applicable , that is, the equation of Langmuir (see Rideal, 1930)

When, however, there are many combining sites of varying adsorptive power and complex aggregates are formed, these simple equations are no longer applicable The Freundlich adsorption isotherm

$$y = KAx^{1/n}$$

where y = amount adsorbed,

x = concentration or pressure of free adsorbate at equilibrium,

A = amount of adsorbent,

K and n are constants, n being greater than 1,

has been found to hold within certain limits in a large number of instances It is probably the result of the even variation of several factors that effect the combination The essential difference between this equation and that given by the law of mass action is that the value of y rises more steeply at low values of x, and, instead of approaching a maximum at high values of x, rises continuously This last feature appears improbable , it would be expected that a saturation value would be reached Modified equations have been evolved which allow for a saturation limit The fact, however, that a combination obeys such a law and not a simple mass action law cannot be regarded as evidence on the nature of the combination but only on its complexity Also the formation of compounds of varying composition cannot be regarded as evidence that we are dealing with adsorption, for similar variation may be found in primary valency compounds if one of the molecules has numerous combining sites , as, for example, in the formation of nitro-compounds of benzene and cellulose

C. Structure of Proteins

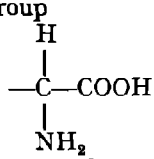
1 *Fibrous Proteins*

By means of X-ray analysis[1] it is possible (1) to detect the existence of crystalline matter even in substances which are apparently amorphous , (2) to determine which way the crystals lie in the body under examination , (3) to calculate the distances at which the pattern, on which the architecture of each crystal is based, repeats itself in the various directions of the structure—the periods of the crystal in these directions ; and, given sufficiently perfect photographs, (4) we can find out the actual positions of the atoms and molecules, and deduce therefrom their size and shape The application of X-ray analysis to cellulose fibres has given results which agree with, and considerably

[1] A clear description of the application of X-ray analysis to the study of fibres is given by Astbury (1933, c).

amplify, the knowledge of their structure obtained by strictly chemical means. The X-ray photographs of fibrous proteins are less satisfactory, but it has been possible to obtain information about their structure from which inferences can be drawn as to the structure of non-fibrous proteins

It is generally accepted that proteins are built up of peptide chains. These can be represented as in Fig VIIA ; R_1 R_2 R_3 represent the tails of amino-acids, the group



being the head. Such a chain need not necessarily be straight, since rotation can occur at each bond

The results of X-ray analysis of the simple protein silk fibroin indicate that it is mainly composed of straight peptide chains lying parallel In particular, from the dimensions of atoms given in Table I, the distance between consecutive " R "s of a peptide chain should be 3 5 Å and the distance " ab " between " R "s on the same side of the chain 7 0 Å Actually the X-ray photograph shows evidence of a periodic repetition of structure at the latter distance In feather keratin the longitudinal distance between two adjacent tails such as R_1 and R_2 is reduced, by slight folding of the chain, to 3 1 Å But the chain can be stretched without rupture until the distance is 3 3 Å ; that is, until the chain is almost quite straight (Astbury and Marwick, 1932)

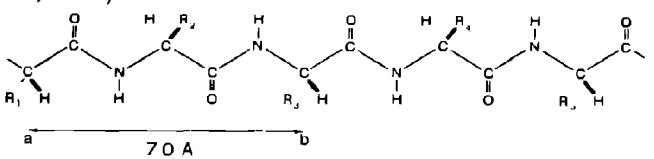


FIG VIIA Straight peptide chain The dotted bonds are behind, the heavy bonds in front, and the light bonds in the plane of the paper

The longitudinal period of unstretched wool keratin found by X-ray analysis is 5 1 Å , this does not correspond with any period in the straight chain Wool, however, will stretch to double its length without tearing , and with stretched wool the period 3 44 Å is found, which agrees almost exactly with that of the straight chain Astbury and Woods (1930) consider that in unstretched wool the chains are coiled up into hexagons, as shown in Fig VIIB, and held in this position by a bond formed between the C and N, with the exchange of an H atom in (Astbury, 1936)

Other proteins give less satisfactory X-ray diagrams There is evidence of a period of about 8 4 Å in stretched gelatin. Astbury (1933, a) suggests that this is the length of three amino-acid residues (possibly glycine, proline, and oxyproline) in a chain so twisted that each " R " lies in the same plane as the third before it.

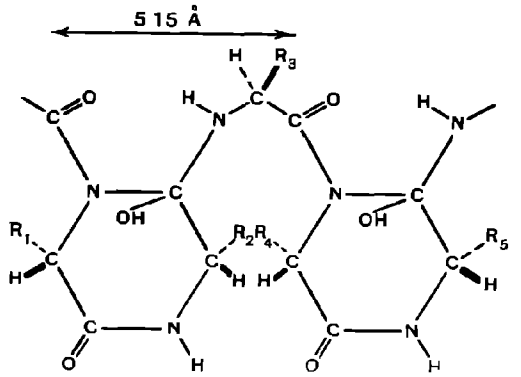


FIG VIIB Peptide chains of keratin in contracted state.

Muscle-fibres[1] in the relaxed state show a period equal to that of unstretched wool, but the fibres cannot be stretched without tearing It is inferred that the chains of muscle-fibres are coiled like those of wool, but that the hexagons so formed cannot be pulled out because they are fixed by some chemical combination In contracted muscle and unstretched gelatin X-ray evidence of a regular structure disappears

Now, unless some fundamental structures other than the peptide chains occur in proteins, the only differences between them must lie in the " R "s The attraction between adjacent " R "s increases with increasing length of the " R "s and with the presence in them of polar groups This attraction tends to bring " R "s in close proximity and parallel, with a consequent crumpling of the peptide chains At the same time long " R "s make a close parallel arrangement of the peptide chains impossible The straightness and close fit of the chains will therefore depend on the nature and order of the " R "s The amino-acids of silk are mainly glycine (40 per cent) and alanine- (25 per cent) The "R"s of these two acids are — H and — CH_3 , there will be little attraction between these, and therefore no tendency to shorten But in proteins with longer and polar " R "s there will be a considerable attraction between them which will tend to distort the chain in order to bring them in contact In the suggested structure of unstretched keratin adjacent " R "s lie parallel and in contact.

In these fibrous proteins the X-ray patterns are not so clear as those of cellulose In part this is due to the fact that the constitution of the proteins is not so simple as suggested Silk fibroin also contains 11 per cent of tyrosine gelatin, 7 per cent of leucine, 6 per cent of glutamic acid, and 14 per cent of diamino acids, and keratin a full complement of amino acids Astbury (1933, b) suggests that the pattern of feather keratin repeats at a hundred amino-acid residues Also it is probable that the constituent chains do not all lie parallel.

[1] Similar X-ray diagrams can be obtained with threads of myosin (Boehm and Weber, 1932).

The peptide chains of these fibrous proteins are considered to be held together by polar attraction between =CO and =NH groups of adjacent chains, by salt formation (or possibly peptide links) between — COOH and — NH_2 groups of dibasic and diamino acids in adjacent chains, by cystine molecules the halves of which lie in adjacent chains; also possibly by peptide chains lying across the general direction They are bound together in parallel chains, some hundreds of chains to the bundle

2 *Non-fibrous Proteins*

(*a*) *Molecular Weights*

In the proteins considered we see a progressive change from the straight chains of silk, through the distorted chains of unstretched keratin, to the patternless unstretched gelatin and contracted muscle-fibre It is probable that differences in the nature and sequence of the " R "s of these proteins determine a varying degree of twisting and crumpling of peptide chains We may infer that other proteins are also composed of peptide chains linked in the same ways, but that a parallel arrangement of these chains is not possible, owing to the crumpling of the chains determined, to some extent, by the nature and sequence of the component amino-acids

An important question then arises, whether or no the peptide chains of such a protein are grouped together in particles of constant size, that is, whether such a protein is to be regarded as having a definite molecular weight or merely as an aggregate of chains, the dimensions of which may vary with external conditions Osmotic pressure measurements can only give values for the average size of particles in a solution, such measurements have, however, shown that the average particle weights of various proteins varied little with conditions This was particularly demonstrated in the case of egg albumin by the classical work of Sørensen (1917), which was expanded by Marrack and Hewitt (1929)

The ultracentrifuge method introduced by Svedberg (see Svedberg, 1930, a) has made it possible to measure the actual sizes of large particles in solution The essence of this method is to subject the solution to an intense centrifugal field, and to measure either the rate of sedimentation (giving the sedimentation constant, S) or the distribution of the particles in height after equilibrium is reached In relatively dilute solutions, in which interfering factors are not too great the particle weight can be calculated from the sedimentation and diffusion constants, or from the equilibrium distribution

A substance in solution is said to be monodisperse when its particles are of uniform size, and polydisperse when they are of various sizes This method has shown that many natural proteins, in solution, are of uniform size, these proteins may therefore be said to have definite molecular weights

The molecular weights (Table III) found by this method agree well with the average particle weights calculated by osmotic pressures and rates of diffusion (Lamm and Polson, 1936)

TABLE III

Sedimentation Constants and particle weights of proteins

Protein	*Sedimentation constant at 20° C* $\times 10^{13}$ (1, 2)	*Particle Weight from Sedn const and Diffn const* (1, 2)	*Particle Weight from Sedn Equilibrium* (1, 2)	*Dissymmetry Constant* (1)	*Approximate Radius of Particle* Å
Lactalbumin	1 9	17,500	—	—	—
Lactoglobulin	3 12	41,800	37,900	1 1	—
Pepsin	3 3	35,500	39,200	1 1	—
Insulin	3 47	40,900	35,100	1 0	—
Bence-Jones Protein α	3 55	—	35,000	1 1	21 8
Bence-Jones Protein β	2 85	37,700	—	—	—
Egg Albumin	3 55	43,800	40,500	1 1	21 7
Carboxyhaemoglobin, Horse	4 5	69,000	68,000	1 2	—
,, ,, Man	4 5	63,000	—	1 2	—
Serum Albumin, Horse	4 5	70,200	66,900	1 2	—
Serum Globulin Horse	7 1	167,000	150,000	1 3	—
Edestin	12 8	309,000	—	1 2	39 6
Excelsin	13 3	294 000	—	1 1	39 6
Thyreoglobulin	19 2	628,000	650,000	1 5	
Haemocyanin Busycon					
Main constituent	99 7	6,680,000	—	1 2	
Aggregation constituent	130 4	9,660,000	—	1 3	

(1) Svedberg and Eriksson-Questel (1935–1936) (2) Svedberg (1937)

A striking feature that has also been brought out is that the proteins can be arranged in groups with particle weights approximately equal to whole number multiples of the lowest particle weight 17,500 This suggests that the protein molecule is built up by the aggregation of definite fundamental structural forms which differ slightly in weight owing to differences in the amino-acids of which they are formed

The proteins show a varying tendency to break up or aggregate without being subjected to drastic treatment Some change usually occurs at pH levels outside a range of stability about pH 4 to 9 These dissociation products may reunite to form a particle of the original size when the reaction is brought back within the stability range. In some instances the dissociation products are simple fractions of the original particle (Svedberg, 1937).

The behaviour of serum proteins is of special interest in immunology. McFarlane's (1935) investigations with the ultracentrifuge have brought out certain remarkable points in connexion with the molecular weights of these proteins For example, horse serum contains about equal amounts of albumin and globulin as estimated by precipitation by half saturation with

ammonium sulphate Albumin so prepared has a molecular weight, in solution, of 70,000 In solutions of globulin, so prepared, the greater part of the particles are uniform and have a weight of about 150,000. But in undiluted serum only about $\frac{1}{8}$ of the protein (G fraction) is in the form of particles equal to those forming the bulk of the separated globulin About $\frac{2}{3}$ of the particles (A fraction) are of the size of the particles of separated albumin The remaining $\frac{1}{6}$ are of intermediate size (X fraction)

When separated albumin and globulin are mixed in the proportions and concentrations found in serum, the ratio of the A and G fractions is the same as that found in serum When horse serum is diluted with 1 per cent. sodium chloride solution the ratio of the G fraction to the total protein increases, reaching the limiting value 36 per cent with a $\frac{1}{4}$ dilution This reversible formation of molecules of G size takes place to a less degree in cow and human sera (Table IV)

TABLE IV

Percentage of serum protein found in the G fraction and in the globulin fraction as estimated by half saturation with ammonium sulphate (McFarlane, 1935)

	G fraction	*Globulin by half saturation*
Horse serum, undiluted	20	49
,, ,, , diluted $\frac{1}{4}$ with 1 per cent NaCl	36	—
Cow serum, undiluted	17	41
,, ,, , diluted $\frac{1}{4}$	28	—
Man serum O D B , diluted $\frac{1}{4}$	6 1	33
,, ,, J S P, ,, $\frac{1}{4}$	7 5	—
,, ,, ,, ,, $\frac{1}{8}$	15	—

It appears, therefore, that the particles of serum globulin break up in the presence of serum albumin, the extent of this process depending on the albumin concentration This remarkable behaviour does not, however, seem to be peculiar to serum globulin Pedersen (1936) found that other proteins (serum albumin and haemoglobin) also break up to a considerable degree in the presence of proteins of smaller particle size or of protamines

It has always been an open question whether eu- and pseudo-globulin could be considered as separate entities present as such in serum When globulin is fractionated with ammonium sulphate the euglobulin formed is polydisperse, with an average particle size considerably above that of unfractionated globulin There is no evidence of such a fraction of high particle size in untreated serum or in unfractionated globulin But it does not follow that the particles of the G fraction of serum and of unfractionated globulin do not differ in chemical composition, molecular structure and in ease of aggregation or dissociation

No great difference in the composition of eu- and pseudo-globulin is shown by the nitrogen partition (Table XIII), but the different fractions of globulin are not chemically identical. In particular, Hewitt (1934) found that the proportion of carbohydrate was higher in fractions precipitated with higher concentrations of sodium

sulphate ; this is the reverse of what could be explained by a progressively greater contamination with albumin The differences in optical rotation found by Hewitt (1927, a) may be due to inter- or intra-molecular rearrangements (comparable to denaturation), and not be dependent on differences of structure or composition in the molecules of the globulin of the original serum

On the basis of solubility measurements Sørensen (1925) concluded that the fractions eu- and pseudo-globulin (E and P) form dissociable compounds, E_mP_n, of varying composition and solubility, and that it is impossible to separate them completely This theory does not appear compatible with the particle sizes found in serum Sørensen's observations would also fit the hypothesis that the fractions are formed from the natural serum globulin by a reversible change

Another question of great importance which is solved by ultracentrifuge methods is that of the shape of protein molecules As seen in Table III some (e g egg albumin) are spherical, while none deviates greatly from a spherical shape The dimensions found show that the constituent atoms are closely packed The polar parts of the molecule are, presumably, on the surface and the non-polar tails of amino-acids in the interior

It is difficult to see how a single peptide chain could be packed into such a compact particle, particularly as the particle is able to spread out to form a thin layer If a series of chains are packed together, why should the naturally occurring sizes be so definite? Recently Wrinch (1937, a) has suggested that a series of amino-acids may be bent into a series of hexagons which are closed in the same way as the hexagonal ring in contracted keratin (the whole forming a closed system) The simplest of such structures is formed by two amino-acids , a regular structure containing 6 amino-acid residues is illustrated in Fig VIIIA Progressively larger molecules with a threefold symmetry can be built up by forming the structure illustrated in Fig VIIIB These symmetrical molecules can also polymerize, e g three 6 cyclol molecules can join up by CO and NH groups (Fig VIIIA)

These structures, which Wrinch calls cyclol molecules, are laminar , on one surface (the front) hydroxyls and the side chains of the amino-acids emerge , on the other side (the back) hydroxyls only Compact bodies can be formed from such laminae, either by curling up one lamina or by piling laminae one on the other Such piled laminae would be joined front to front by the $-S=S-$ bridge of cystine molecules, which have their $-C(NH_2)COOH$ parts in both laminae, or back to back, by hydroxyl links between OH groups One type of curled up closed lamina can be formed by a series of cyclols, containing $72,288,648,\ldots 72n^2$ amino-acids (Wrinch, 1937, b), this may be the basis of the limited groups of molecular weights actually found

This theory appears compatible with the facts known about the behaviour of proteins and affords a concrete picture of their structure on which to base our ideas.

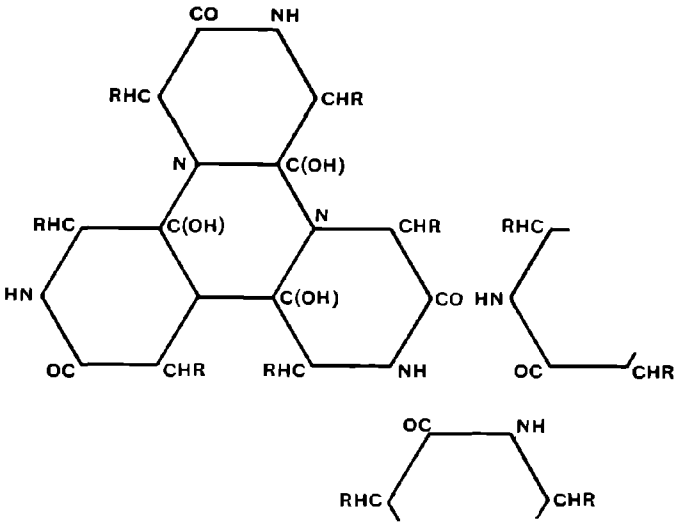


FIG VIIIA A " Cyclol 6 " molecule

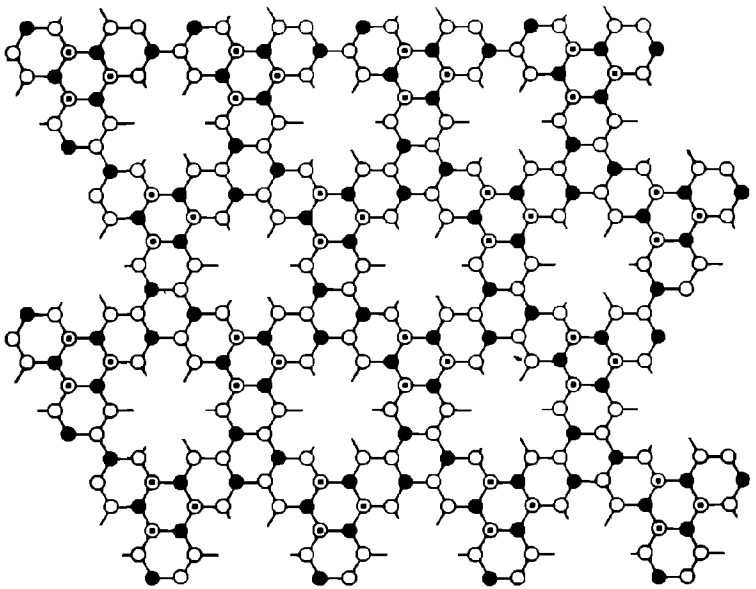
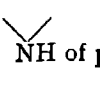


FIG VIIIB from Wrinch (1937)
Reproduced by kind permission of the Royal Society.

The study of fibrous proteins suggests that proteins may be built of groups of amino-acids, periodically repeated For example, in the egg albumin molecule a group containing one lysine, arginine, methionine and 3 glutamic acid residues may be repeated 12 times However numerical considerations show that such repeated groups cannot be identical Egg albumin, for example, contains only 4 cystine (or cysteine) residues in the molecule and can therefore be broken into only four wholly similar parts On the other hand, Bergmann and Niemann (1937) suggest that each amino-acid recurs at regular intervals along a peptide chain If this is so, " nodes " must occur at which groups of amino-acids recur together Thus in egg albumin, the group methionine, lysine, arginine and glutamic acid will recur at intervals equal to $\frac{1}{12}$ of the total chain length But because other amino-acids recur at intervals of different lengths, (e g aspartic acid, $\frac{1}{18}$ of the chain length) such nodes would not recur in exactly the same environment of other amino-acids In the proposed cyclol molecule of Wrinch, which provides a two-dimensional arrangement, those amino-acids that occur in the molecule more frequently may be supposed to be grouped around those that occur less frequently But in this plan also the environments of the different groups of these more frequent amino-acids would not be uniform

The molecular weights given in Table III are those for proteins prepared with the minimum of manipulation We cannot regard the molecular weights of proteins as fixed and unchanging in the same way as those of alcohol or cane sugar , proteins may be made polydisperse with little apparent change in their properties Nevertheless natural proteins appear to occur in particles of definite and constant size The particle weights in solution of the azo-proteins described in Chapter III may not be uniform and may differ greatly from those of the proteins from which they are prepared Nevertheless there is no reason why their general structural plan should be altered essentially We may therefore think of the proteins with which we shall have to deal as compact particles, which present a varied surface containing such polar groups as

$-COOH$ or COO^-, $-NH_2$ or $-NH_3^+$, $HN{=}C\begin{smallmatrix}NH-\\ \diagup\\ \diagdown\\ NH_2\end{smallmatrix}$ of arginine,


NH of proline, HN N of histidine,

—OH of tyrosine, besides the = CO and = NH of the peptide chain, and any polar groups that may be attached artificially It is possible that proline and oxyproline may be of peculiar importance Since the nitrogen atom of these amino-acids forms part of a hexagonal ring, a kink must occur wherever a proline or oxyproline residue enters into a peptide chain The shape of the molecules can probably be appreciably altered, by stretching coils or pulling apart polar links, without serious change of their surface structure.

(*b*) *Surface Films*

Lee and Wu (1932) and Hughes and Rideal (1932) have obtained films of egg albumin, serum albumin and globulin, glutenin and gliadin on water In their work precautions were taken to ensure that the protein remained on the surface, which had not been done in the previous work of Gorter and Grendel (1926) The weight of protein ($0{\cdot}36 \times 10^{-7}$ g) per sq. cm. in the thinnest films formed indicated that the peptide chains and the " R " tails lay in the water surface. When such films were compressed the area occupied was reduced to one corresponding with an arrangement with the peptide chains in the water surface, but the " R "s forced off the surface, like the hydrocarbon tails of fatty acid molecules in a compressed film , the thickness of such films is about 12 Å Wrinch (1937) finds that the weight per area of these more compact films is compatible with the " cyclol " structure The spreading out of the approximately spherical protein molecules into a flat sheet under the influence of the polar forces of the water surface suggests that the constituent peptide chains of protein molecules are connected in the compact form mainly by polar bonds It recalls the distortion of pentaerythritol tetrapalmitate molecules on a water surface (p 17)

Egg albumin is denatured and becomes insoluble when it forms surface films The molecules that have been spread out as described do not revert to their original form when they escape from the surface However, serum proteins are not denatured in this way (Wu and Ling, 1927) This return from a flat to a globular form is less surprising if the formation of a surface film involves only the flattening of a lamina or separation of laminae, than if it involves the rearrangement of the molecule into a layer of parallel peptide chains

(*c*) *Solubility and Hydration*

The behaviour of proteins on the surface of water indicates that it is the polar =CO and =NH groups of the peptide chains that are chiefly attracted by water Presumably the solubility of proteins also depends mainly on these groups The acid and basic groups, which only amount to about one gramme equivalent of each per 1,000 g of protein, cannot be sufficient to keep the molecules in solution The polar groups of the peptide chains may be packed in the interior of the molecule, as in the fibrous proteins, or turned towards the surface Variations in the solubilities of proteins will depend on the accessibility of these groups to water, and the degree to which they neutralize each other when in proximity (as in ortho-phenol derivatives, p 20)

The water molecules attracted by polar groups are not available for the solution of other molecules and increase the apparent volume of the hydrated molecules The proportion of polar to non-polar groups in proteins is not high , it would therefore not be expected that they should be highly hydrated Adair and Robinson (1930) conclude from their osmotic pressure measurements that the volume of

hydrated serum globulin is about 1 3 c c per g This would result from a layer of water about two molecules deep over the whole globulin molecule The negligible effect of proteins on the solubility of inert gases (Stoddart, 1926–7) also indicates that the degree of hydration of proteins is not high

(d) Acid and Basic Properties

Protein molecules contain groups which can function as acids and bases, that is, can lose or take on a hydrogen ion and thereby acquire a negative or positive charge The titration curve of egg albumin with the pH ranges over which, according to Simms (1928), the various groups change from the un-ionized to the isolated state is shown in Fig IX A serum globulin molecule contains about one hundred

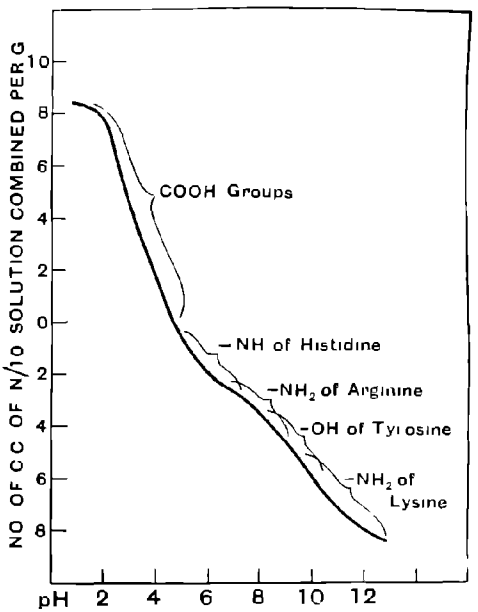


FIG IX Titration curve of egg albumin, according to Simms (1928), showing pH range over which the degree of dissociation of various groups changes [1]

and forty of such acid and basic groups , they are chiefly the —COOH groups of di-carboxylic acids and —NH_2 groups of lysine (Fig IX). It is generally considered that the dissociation constants of these two groups are relatively high, so that at the isoelectric point both are nearly completely ionized On the acid side the effect of increased hydrogen-ion concentration is to convert —COO— into —COOH , while on the alkaline side diminished hydrogen-ion concentration (or increased hydroxyl-ion concentration) changes —NH_3^+ to —NH_2

[1] The dissociation of the —OH group of tyrosine changes over a range with centre at pH 10 2 and not as given by Simms

As a result the protein molecule as a whole has a negative charge on the alkaline side and a positive charge on the acid side; while at the isoelectric point it is neutral As almost all work on immunity is done on the alkaline side of the isoelectric points of the proteins concerned, they may be regarded as negatively charged

$$\underset{\text{On the acid side}}{HOOC-X-NH_3^+} \leftarrow H^+ + \underset{\text{Isoelectric Protein}}{^-OOC-X-NH_3^+} + OH^- \rightarrow \underset{\text{On the alkaline side}}{^-OOC-X-NH_2} + H_2O$$

The highly polar $-COO^-$ and $-NH_3^+$ groups apparently largely neutralize each other at the isoelectric point, hence the minimum solubility of proteins at this point This neutralization is quite possible as the groups, if evenly distributed over the molecule, are only about 7 Å apart

(e) Denaturation

Various treatments, such as with heat and alcohol, can reduce the solubility of proteins in water The change appears to be a molecular rearrangement, denaturation by heat runs the course of a monomolecular process (Chick and Martin, 1910). The process of denaturation must be distinguished from the secondary process of coagulation, which does not take place in the absence of salt, except at the isoelectric point By methods of X-ray analysis Astbury and colleagues (1935) have found evidence that some proteins, when denatured, are composed of extended peptide chains arranged in parallel bundles Such a change to a fibrous structure would account for the loss of solubility It might also account for the change in reactivity of $-S-H$ and $-S=S-$ groups studied by Mirsky and Anson (1934) The change of optical activity that occurs on denaturation (Pauli and Weiss, 1930), indicates that the backbones of the peptide chains are involved, since the asymmetric C atom is in the peptide chain As the process is not readily reversible it is probable that the new structure becomes fixed by chemical bonds

It appears that there may be different degrees of denaturation, McFarlane (1935) considers that alcohol, even in the cold, causes an early stage of denaturation, and Astbury and colleagues (1935) found that a fibrous structure appeared in crystals of egg albumin and pepsin on drying, this they regarded as a stage of the same process as gives rise to complete denaturation It is possible that the change that takes place in serum on heating for ½ hour at 55° C is also an early stage of protein denaturation The solubility of the globulin in serum so heated is reduced and the viscosity of the serum is increased (Holker, 1921; de Nouy, 1929), although the solutions remain clear However, McFarlane (1935) found that heating for 2½ hours at 56° C produced little change in the sedimentation constants of the serum proteins

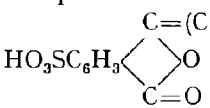
(f) Adsorption by Proteins

Fibrous proteins may adsorb molecules either on the surface of bundles of peptide chains, or between the chains The latter process, however, can only take place if the adsorbed molecules are small

enough to penetrate between the chains, not larger than the lower fatty acids In the case of non-fibrous proteins the same two kinds of adsorption may occur, but since only small molecules can penetrate between the peptide chains we shall be mainly concerned with adsorption on the surface

This adsorption is not merely a matter of opposite charges If this were so there would be no selection between the adsorption of, for example, different positively charged molecules or particles by negatively charged proteins, nor between the adsorptive power of different negatively charged proteins for positively charged molecules, also such proteins should not absorb negatively charged substances Actually considerable differences are found in the degree to which similarly charged substances, dyes for example, are adsorbed by the same protein, and in the degree of adsorption of the same substance by different proteins Also negatively charged proteins can adsorb negative charged dyes, for example, eosin is adsorbed by protein when the pH is well above 9, this may be seen by the change of colour and loss of fluorescence

Nevertheless, the repulsion between similarly charged particles reduces the number of collisions and therefore reduces the degree of adsorption, hence the degree of adsorption varies with the pH This is well illustrated by the quantitative studies of Grollman (1925) on



the adsorption of phenolsulphonephthalein (Fig X) At pH levels above 8, both $-SO_3H$ and $-OH$ groups are dissociated and therefore both ends of the phenolsulphonephthalein molecule are negatively charged Little adsorption therefore takes place to proteins, as these are also negatively charged at this pH At lower pH levels (5–7), still above the isoelectric point of the protein, the $-OH$ groups are in the undissociated form and this end of the molecule is available for adsorption on the protein, although both dye and protein are still negatively charged The maximum adsorption occurs on the acid side of the isoelectric point, when the charge on the protein is positive Serum globulin adsorbs less than serum albumin at pH above 7, although its negative charge is less, showing that the electric charge is not the sole factor

Salts may alter the degree of adsorption by affecting the charges on protein or adsorbate Thus the adsorption of nucleic acid (negative charge) by proteins at pH 7 3 only occurs in the presence of salt (Przylecki and Grynberg, 1933), here reduction of like charge favours adsorption On the other hand, the adsorption of rose bengal (negative charge) by proteins (positive charge) on the acid side of the isoelectric point is reduced by salts (Rosenthal, 1926), here reduction of unlike charges reduces adsorption

It is difficult in many cases to distinguish between adsorption and formation of chemical compounds Hewitt (1927, b) considers that

compounds are formed between the basic groups of proteins and phthalein dyes, similar to those formed between these dyes and heavy metals or simple nitrogenous bases

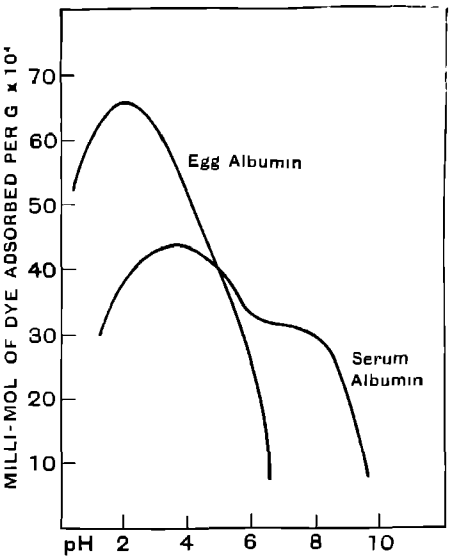


FIG X Effect of pH on the adsorption of phenolsolphonephthalein by egg and serum albumin (Grollmann, 1925)

(g) *Adsorption of Proteins*

The work of Jones (1928, b) demonstrates that proteins are adsorbed on special patches of collodion particles He showed by immunological methods that collodion particles will firmly adsorb five different proteins, none interfering with the adsorption of the others It appears that each of these proteins must be adsorbed on patches of the particles at which the spatial arrangements of the polar groups are suited to the spacing of the polar groups on the protein It can be said that the patches adsorb the various proteins specifically, although the whole particles do not

It is not necessary that a solid particle should be involved in such a process , proteins may be bound specifically in the same way to suitably spaced groups on large molecules in solution In such a case either the protein or the other molecule may be considered the adsorbent The adsorption of proteins may in some cases be dependent on differences in the sign on the charges of the protein and adsorbent In the case of the adsorption of egg albumin and gelatin by collodion particles this is not so, as was shown by Hitchcock (1926) The adsorption is maximum at the isoelectric point (Fig XVII) and falls off on either side, although on the acid side the protein acquires a positive charge (opposite to that of collodion) and on the alkaline side

a negative charge In this case addition of salt increases the adsorption in acid solution, although the charges of adsorbent and protein are opposite The adsorption obeys Langmuir's adsorption isotherm' not the Freundlich equation

(h) Lipins[1] *and Proteins*

The relation of lipins to proteins is of some importance in connexion with immunology Numerous workers (e g Theorell, 1926, 1930) have found cholesterol and phosphatides in the protein fractions precipitated from serum This in itself is of no great significance as these lipins are themselves precipitated by the salting-out methods used for separating protein fractions Gardner and Gainsborough (1927) found that a considerable part of this cholesterol was left behind on the filter papers in the process of further purification of the fractions, but that the euglobulin retained a much larger amount than the other fractions (16 6 mg per g of protein) Chick (1914), finding more phosphatide in euglobulin than in pseudoglobulin, suggested that euglobulin was formed from pseudoglobulin by combining with lecithin There is no doubt that the lipins in serum are held in suspension by the proteins The question arises whether they form a part of the structure of serum proteins, particularly euglobulin, or are merely adsorbed on the surface of proteins or protected by a protein layer Serum albumin crystallizes as readily from serum from which the lipins have been extracted by the method of Hardy and Gardiner as from untreated serum (Young, 1922) With regard to euglobulin the reports differ The proteins prepared from serum by the Hardy and Gardiner method dissolve in distilled water to form a clear solution (Young, 1922), and euglobulin is not precipitated by ordinary dialysis (Jukes and Kay, 1932, working on chicken serum) However, a fraction of the globulin is precipitated from extracted serum by one-third saturation with ammonium suplhate, and Hewitt (1927, a) found this fraction to be insoluble in distilled water Reiner and Reiner (1932) found that fractions of globulin were precipitated from extracted sera by electrodialysis, and by dilution with distilled water at pH levels below 6 8 Went and Kuthy (1934) fractionated sera by Howe's Na_2SO_4 method , they found that the " globulin " fraction formed a considerably larger proportion of the total protein of extracted sera than of the protein of non-extracted sera Horsfall and Goodner (1935) found that the peculiar globulin of anti-pneumococcal horse sera no longer exhibited its characteristic solubility relations after lipins had been extracted, only a small precipitate formed when the serum was diluted ten-fold with distilled water Attempts have been made (Went and Farago, 1930) to show that the addition of lecithin to serum increases the amount of the protein precipitated as euglobulin, but the results have been

[1] The word, lipin, is here used to include substances extracted by alcohol and ether—sterols for example—besides those which yield fatty acids on hydrolysis.

neither striking nor consistent Went and Kuthy (1934) found that the changes produced by extraction were not reversed by the addition of suspensions of lecithin and cholesterol

The ultracentrifuge method has shown (McFarlane, 1935) that a very striking change occurs in serum on extraction by the Hardy-Gardner method, a considerable amount of the protein becomes polydisperse It is, at present, not possible to decide whether this is due to the actual removal of lipins, or to the action of alcohol used in the process, quite apart from the lipin extraction

It may be supposed that the polar part of a lipin is attracted to the polar parts of proteins (mainly in the peptide chain backbones) while the non-polar parts are attracted by the non-polar tails of the amino-acids Three possibilities may be considered (1) the lipin may be absorbed on the surface of a globular protein molecule, with its non-polar part towards the water and polar part towards the protein molecule, in this case the solubility of the protein is decreased (2) the protein may form a film on the surface of a clump of lipin molecules, forming relatively large particles (3) the non-polar parts of the lipins may be attached to the amino-acid tails of a globular protein, while the polar part lies on the surface of the protein, in this case the lipin is built into the structure of the protein. Lacunae occur in the cyclol structure of Wrinch through which the polar parts of lipins could reach the surface The solubility of the protein would not necessarily be altered

D Stability of Suspensions

1 *Hydrophobe and Hydrophil Suspensions*

The conditions affecting the stability of suspensions is discussed here in order to avoid interruption in later chapters

If small particles[1] are dispersed in water and do not aggregate, they will sink extremely slowly, and eventually, owing to their thermal agitation, distribute themselves like gas molecules in the earth's atmosphere—the concentration decreasing with the height If, however, such particles aggregate to form relatively large particles the rate of sinking is much increased[2] and the final distribution approaches that of a compact layer at the bottom of the water The particles therefore settle only if they aggregate, they will aggregate if the rate of collision exceeds the rate of separation of particles that have collided

The aggregation of particles is prevented by (1) polar groups on the surface of the particles which attract a layer of water molecules, which must be displaced before the particle can collide, (2) like electric charges on the particles sufficiently high to prevent all particles but those of the highest kinetic energy approaching The aggregation is assisted by (1) strong mutual attraction between the

[1] By " particle " may be meant a single molecule or a group of molecules

[2] A particle, of the specific gravity of protein, of radius 2μ or 20,000 Å will sink about 1 cm in an hour

particles, (2) opposite electric charges A suspension in which the mutual attraction of the particles exceeds their attraction for water (a hydrophobe suspension) depends for its stability on the electric charge of the particles, the particles aggregate and precipitate if their charges are reduced Hydrophil suspensions, in which the attraction of the particles for water is greater than their mutual attractions, are independent of their electric charge

In actual practice, in dealing with the stability of suspensions, what is employed is not the charge on the particles, but the " surface potential " or " electrokinetic potential " This is a calculated value, based on certain assumptions, for the potential difference between the bulk of the water and some apparently immobile layer on the surface of the particle It is estimated from the rate of movement of the particle in a potential gradient The relation of this " surface potential " to the stability of a suspension may be illustrated by the behaviour of a " lyophobe " suspension of fine collodion particles (Fig XI, Loeb, 1922–23, a), similar effects are obtained with air bubbles or oil droplets These suspensions are stable when the surface potential is above about 16 mvt In distilled water the surface potential lies

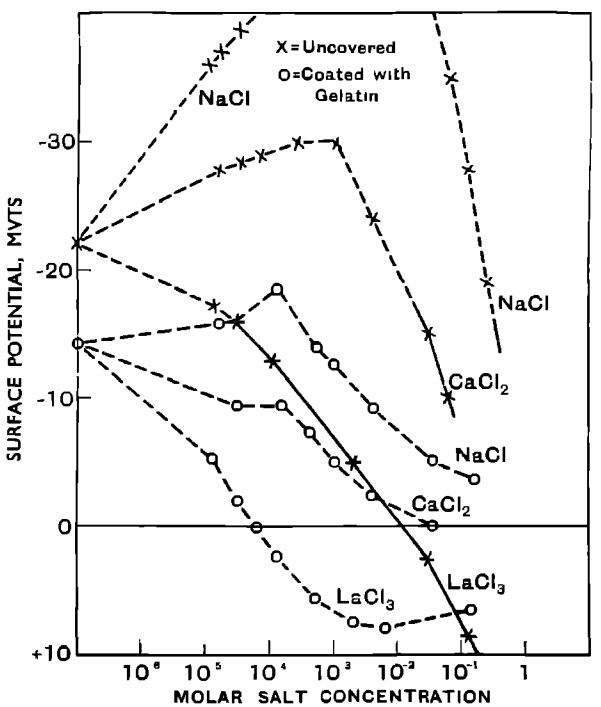


FIG XI Effect of salt concentration on the surface potential and suspension stability of collodion particles uncovered and covered with gelatin (Loeb, 1922–3, a and b) Unbroken line agglutination

between 20 and 30 mvt On the addition of salts of mono- or divalent kations the surface potential rises to a maximum at a concentration of about N/250 to N/1000 and then falls The higher the valency of kation the more rapid the fall The surface potential of the particles falls below the critical level of 16 mvt , and the particles aggregate and settle in the presence of N/2 NaCl, N/16 $CaCl_2$, and N/682 $LaCl_3$

Loeb (1923–4) found little difference between the " critical levels " necessary for the stability of suspensions of various particles (collodion, mastic, graphite, gold) Nevertheless, the levels must depend in part on the relation between the attraction of the particles for water and their mutual attraction, and therefore vary with the nature of the surface of the particle Thus Eagle (1930, c) found the critical potential of the Wassermann antigen to be 2 to 5 mvt Particles of denatured egg albumin at pH 5 8 apparently have a greater attraction for water than have collodion particles, for a suspension remains stable until the surface potential has fallen to 8 mvt (Loeb, 1922–3, c) , but the concentrations of salt required to produce this fall of potential are lower (e g N/128 NaCl) At pH levels further removed from the isoelectric point, pH 3 or 11, higher salt concentrations are required in order to reduce the higher charge of the particles and sodium chloride is ineffective in any concentration Eagle (1930, b) found heat-denatured serum globulin, presumably about pH 7, was aggregated and completely precipitated by N/50 NaCl and N/400 $BaCl_2$

Precipitation of hydrophobe suspensions, as a result of reduction of the charges of the particles, can also be produced by suspensions of opposite charge, as well as by salts

Organic substances, dyes for example, which are adsorbed on the surfaces of particles may also affect the stability of their suspensions In the first place they may have effects similar to those of inorganic ions , owing to their ready adsorption they may be, like polyvalent inorganic ions, effective in very dilute solutions Other substances which have non-polar and strongly polar parts form a layer on the surface of hydrophobe particles, with the non-polar part towards the particle and the polar part towards the water, and thereby give the suspension a hydrophil character , its stability becomes independent of the charges on the particles The familiar instance of this is the " protective " action of various substances, proteins, gums, starch, &c , on gold sol

Besides these obvious effects a hydrophil organic substance may have an opposite effect, even on hydrophil suspensions, which cannot be ascribed to neutralization of electric charges Thus tannin, which is negatively charged, makes agar (also negatively charged) susceptible to precipitation by salts, and greatly increases the sensitivity of negatively charged alkali blue and benzazurin to salts (Brossa, 1923) In these cases it is supposed that the more polar part of the tannin is attracted to the agar or dye, and that the less polar part is turned towards the water

2 *Effect of Proteins on the Stability of Suspensions*

The usual action of proteins on the stability of suspensions is a combination of sensitization and protection, which effect predominates, depends on the concentration of protein and on the pH This is well illustrated by the influence of gelatin on the stability of suspensions of colon bacilli (Table V) At pH levels between 3 and 3 5 the negative charge of the bacteria is reduced by low concentrations of positively charged gelatin and the stability of the suspension is reduced As the concentration of gelatin is increased the increasing amount adsorbed gives the bacteria an increasing positive charge and

TABLE V

The effect of gelatin concentration and pH on the stability of suspensions of B coli *in 0 025 M salt solutions* (Eggerth and Bellows, 1921–2)

	Concentration of gelatin					
	$2\ 5\times10^{-3}$	$2\ 5\times10^{-4}$	$2\ 5\times10^{-5}$	$2\ 5\times10^{-6}$	$2\ 5\times10^{-7}$	0
pH 5 3	—	—	—	—	—	—
5 0	—	+	—	—	—	—
4 7	+	×	—	—	—	—
4 4	—	×	—	—	—	—
4 1	—	×	×	—	—	—
3 8	—	+	×	—	—	—
3 5	—	—	×	—	—	—
3 2	—	—	×	+	+	+
3 0	—	—	+	—	—	—
2 7	—	—	—	—	—	—
2 4	—	—	—	—	—	—

× Agglutination in 1 hr
\+ ,, 24 hrs

renders the suspension stable again At higher pH levels 4 to 4 5 the negative charge of the bacteria is higher and positive charge of the gelatin lower, larger amounts of gelatin are therefore necessary to render the suspension unstable In the highest concentrations the gelatin forms a complete layer on the surface of the bacteria, the stability of the suspensions is consequently independent of the surface potential—compare the protection of collodion particles by gelatin (Fig XI)—except at the isoelectric point (pH 4 7) At this point, as the number of polar groups of the gelatin free to attract water is low, the suspension is still unstable Similar results are obtained with other proteins and other suspensions—mastic, gum benzoin, collodion—but with these other suspensions higher concentrations of proteins are required to produce an effect The great dilution of the concentrations of gelatin and edestin that will affect the stability of suspensions of colon bacilli (Eggerth and Bellows, 1921–2) and the dilutions of normal serum that affect the surface potential of typhoid bacilli (Northrop and de Kruif, 1921–2, a, Fig 21) are remarkable The amounts of protein present, if completely adsorbed, may be sufficient to form a continuous layer of minimum thickness over the bodies of the bacteria, supposing these to be some 3 by 0 5μ, but hardly

enough to cover all the flagellar surface[1] It is therefore probably sufficient that only localized patches of the bacteria should be covered in order that the stability of the suspensions should be affected

The peculiar action of tannin is also shown in the mutual precipitation of tannin and protein solutions Although the optimum pH for the precipitation of protein solutions by tannin is below the isoelectric point of the proteins, precipitation of serum proteins occurs at pH levels as high as 7 5, at which level the proteins are negatively charged

Euglobulin has a sensitizing action on suspensions of both positive and negative particles (Brossa, 1923), comparable to the effect of tannin , it precipitates negatively charged gum benzoin suspensions even when the pH is as high as 8 1 (Wolf and Rideal, 1926), i e although the euglobulin is also negatively charged Globulin preparations also counteract the protective action of serum albumin A similar antagonism between the effects of globulin and albumin can also be demonstrated with unfractionated sera, and is familiar in connexion with the aggregation of red blood corpuscles, which is the basis of the sedimentation test This appears to indicate the presence, preformed in unfractionated serum, of certain globulin molecules specially liable to aggregate and precipitate

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[1] Abramson (1930) also found that serum would affect the surface potential of quartz particles and oil droplets in concentration as low as $1/10^8$ In the absence of data as to the size and concentration of these particles it is impossible to calculate how much of their surface could have been covered

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CHAPTER II

THE NATURE OF ANTIBODIES

Controversy concerning the nature of antibodies[1] centres round the question whether they are proteins or not This question is not only of great practical importance but is also essential to a theoretical discussion of the mechanism of immunity reactions The properties of antibodies will therefore be discussed mainly from the point of view of the information they may give on this point

A Separation of Antibodies from other Serum Constituents

Attempts to isolate antibodies have been mainly directed towards reducing the ratio of the antibody titre to the protein concentration (the "concentration factor") of the resulting products Owing to the clinical and theoretical value of such purification the matter has been investigated very thoroughly

The methods used have been (1) non-specific methods—fractional precipitation of serum, and adsorption , and (2) specific methods depending on precipitation in combination with antigen and extraction from the compound

1 *Non-specific Methods*

(*a*) *Variation of Salt Concentration*

It is generally agreed that antibodies are precipitated with the globulin fraction of serum, whatever method of precipitation is used The belief that the serum globulin consisted of two or more separable components led naturally to the supposition that antibody would be found in one such fraction and to attempts to separate out the active fraction by the usual methods—precipitation with high concentrations of salt (especially ammonium sulphate), dialysis, electrodialysis, and dilution and acidification [2] Although in some

[1] The following definitions are given by Topley and Wilson (1936) —

An Antigen is any substance which, when introduced parenterally into the animal tissues, stimulates the production of an antibody, and which, when mixed with that antibody, reacts with it in some observable way

An Antibody is any substance which makes its appearance in the blood serum or body fluids of an animal, in response to the stimulus provided by the parenteral introduction of an antigen into the tissues, and reacts specifically with that antigen in some observable way

[2] Reports on the distribution of antibodies are very conflicting This is partly due to the different methods employed for separating eu- and pseudo-globulin In reviews of the voluminous literature the results are usually quoted without mention of the method Small differences of technique, also, may affect the results considerably The amount of globulin precipitated from a serum on dialysis depends on the completeness with which salts are removed , the effect of the degree of salt removal on the amount of antibody precipitated is well illustrated by the experiments of Coulter (1927) on haemolysin On the other hand Hartley (1914, a) found that the antibody to rinderpest was precipitated when the serum was dialysed against tap water but not when it was dialysed against distilled water Selter (1927–8) found that antibodies to lipins were precipitated on dilution and acidification at 0° C , but not at 37° C

cases a sufficiently high proportion of antibody may be contained in a particular globulin fraction to make its separation worth while for clinical or other purposes, antibodies have not as a rule been found to be confined to any particular fraction Since it is uncertain that the fractions are not artefacts this is not surprising

The distribution of antibodies in the fractions separated by ammonium sulphate has been most thoroughly studied in the case of diphtheria antitoxin, but the conclusions appear applicable to antibodies in general The antitoxin is not confined to any one fraction (Banzhaf and Gibson, 1907 , Barr and Glenny, 1931 , Barr, Glenny and Pope,

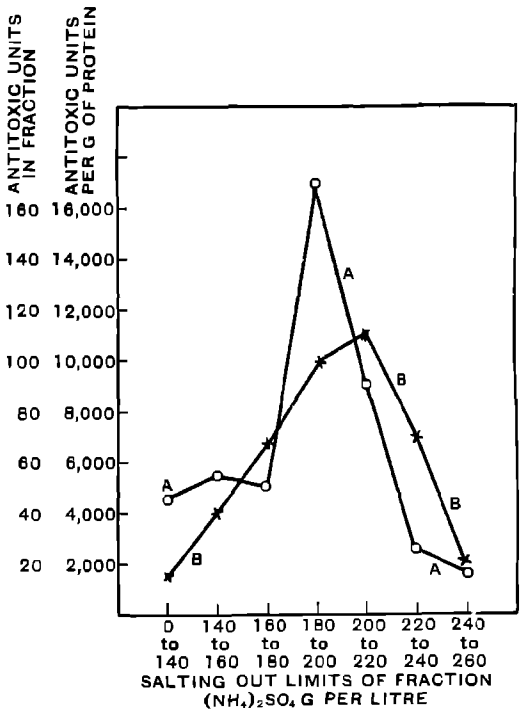

FIG XII Distribution of diphtheria antitoxin in fractions precipitated with ammonium sulphate Original serum 625 units per c c (Barr and Glenny, 1931) (A) No of antitoxic units precipitated in each fraction (B) No of units per gramme of protein in each fraction

1931 , and Barr, 1932) (see Fig XII) Great variation in the distribution among the different fractions is found in the sera of different species (Barr, 1932), and even in the sera of the same animal examined by the same method at different times (Gibson and Collins, 1907 , Ledingham, 1907) Barr, Glenny, and Pope (1931) found that added

euglobulin and albumin, derived from sera containing no antitoxin, shifted the maximum precipitation of antitoxin respectively towards the lower and higher ammonium sulphate concentrations These results agree with Sørensen's (1925) views on the fractionation of serum globulin

Removal of the water-insoluble euglobulin by dialysis gives better results in the case of diphtheria antitoxin of horse serum, only a small proportion is lost with the euglobulin [1] However, Barr (1932) found a large amount of antitoxin in the water-insoluble euglobulin of the sera of other species (e g cow), and even in horse serum the confinement to the pseudoglobulin fraction is peculiar to diphtheria antitoxin Addition of normal euglobulin increased the amount of diphtheria antitoxin precipitated in the water-insoluble euglobulin fraction of horse serum According to Jukes, Fraser and Orr (1934) antitoxin is found associated with the livetin fraction of the eggs of fowls immunised with diphtheria toxin Livetin is chemically and immunologically similar to fowl serum globulin (Jukes and Kay, 1932)

(*b*) *Precipitation with alcohol*

Antibodies may be precipitated, together with protein, by alcohol, and are not damaged if precautions are taken to ensure that the proteins are not denatured in the process This may be done either by working in the cold (Mellanby, 1908) or by bringing the alcohol concentration promptly up to over 90 per cent (Merrill and Fleisher, 1932) Mellanby found that the precipitation of diphtheria antitoxin ran parallel with that of protein Moloney and Taylor (1928), using various alcohol concentrations at different pH levels, were unable to obtain a " concentration factor " more than, approximately, 30 per cent above that of untreated plasma

It is rarely possible to obtain a fraction, by any of these means, which both contains a large proportion of the antibody and has a concentration factor more than two or three times that of the untreated serum In some cases a fraction may be obtained which contains a small proportion of the antibody in a state of considerably greater purity, always, however, it contains abundant protein

However, the antibodies in certain horse[2] sera occupy a peculiar position, in that a large proportion of the antibody can constantly be separated, highly purified, in a fraction of the serum globulin. Although precipitation with ammonium and sodium sulphate (Avery, 1915, Felton, 1928) has proved no more satisfactory than with other antibodies, they have been very efficiently purified by methods

[1] Ornstein (1928) found that euglobulin separated by electrodialysis contained 36 per cent of the total antitoxin Precautions were taken against denaturation in the process, but some may have occurred Denaturation of the globulin during the electrodialysis is the explanation of the complete precipitation of the antitoxin by electrodialysis found by Adolf (1924), as in this case all the globulin was precipitated

[2] Not, however, those of rabbit sera (Heidelberger and Kendall, 1933)

based on precipitation in low salt concentration and with alcohol. The first method (Felton, 1926) depends on the fact that the globulin which contains the antibodies is not precipitated at moderate salt concentration (N/40) at the usual optimum pH about 5 2, and is precipitated at lower salt concentrations (N/200) the optimum pH being about 6 7 In the alcohol method (Felton, 1931) the antibody is precipitated in the cold with low concentrations of alcohol (10 per cent) , the precipitate can be redissolved and further purified by precipitating inactive proteins at pH 5 in N/40 salt solutions By these means some 90 per cent of the inactive proteins can be removed Even purer preparations can be obtained by precipitation with $AlCl_3$ or $ZnCl_2$ (Felton, 1932) More recently Murdick and Cohen (1935), using similar methods, have removed 60 to 90 per cent of the inert proteins from horse anti-meningococcal sera with little loss of antibody

(c) *Adsorption*

The successful purification of enzymes by adsorption in the hands of Willstatter and his school suggests that antibodies might be purified by the same means Eisler and Spiegel-Adolf (1929) studied the adsorption of antibodies by aluminium hydroxide and elution with N/100 NaOH They found that with typhoid agglutinin a concentration factor six times that of the untreated could be reached Subsequent studies of the adsorption and elution of serum proteins (Spiegel-Adolf, 1932), showed that albumin was adsorbed more easily than pseudoglobulin while euglobulin was not eluted The purification had been due in part to this, although the agglutinin is usually mainly found in the euglobulin fraction With other antibodies purification by this method was not successful

Olitzki and Frankel (1931) described the adsorption of various antibodies with kaolin, followed by elution with glycine in sodium chloride solution An eluate was obtained which would agglutinate typhoid bacilli in a dilution of 1/1,000, but 1 c c of this would not produce anaphylactic shock in guinea-pigs sensitized with proteins of the same serum Presumably it contained less than 0 6 mg of protein in 1 c c Frankel (1932) described eluates prepared by this method from diphtheria antitoxin and typhoid agglutinin (flagellar) These eluates contained from 12 to 24 per cent of the antibody in the original sera, but did not give the Esbach and Spiegler tests for protein Dr C G Pope (private communication) and the reviewer (unpublished) failed to confirm these results , the concentration factor remained unaltered Subsequently Olitzki (1932) described the purification of the somatic-agglutinin for *B enteritidis* Gartner by this method Only the weakest eluates failed to give a positive test with Esbach's reagent Silber and Demidowa (1932) failed to confirm these results Rosenheim (1935), after numerous attempts, concluded that there was no indication that the flagellar and somatic typhoid agglutinins can be eluted specifically and free from protein after adsorption on kaolin or aluminium hydroxide

(d) Cataphoresis

Michaelis and Davidsohn (1912) found the isoelectric point of agglutinins determined by cataphoresis to lie between pH 5 3 and 6 0, Szent-Gyorgi (1921) narrowed the limits to 5 4–5 7 These limits agree well with the various estimates for serum globulin (Rona and Michaelis, 1910) The antibodies therefore migrated with the serum globulin [1] Girard and Louranc (1934), studying untreated sera, found that agglutinin for *B paratyphosus B* and haemolysin for sheeps' red blood corpuscles moved to the anode at pH 5 2 the greater part of the protein of the sera moved to the cathode At pH 6 3 the antibodies did not move to anode or cathode As Girard and Louranc did not try any intermediate pH it is not possible to fix the isoelectric points of these antibodies, but they appear to have been on the alkaline side of that of normal serum globulin As, however, globulin fractions, associated with antibody, which have, such alkaline isoelectric points have been separated from anti-sera, this is no argument against the protein nature of antibodies

2 *Methods based on Specific Precipitation*

Since antibodies combine specifically with antigens and are removed from the serum if precipitation or flocculation occurs, it should be possible to recover a highly purified antibody from the antigen-antibody compound Various attempts have been made to recover antibodies in this way, especially from combination with particulate antigens such as bacteria and red blood corpuscles, only the more recent of these will be discussed

Locke, Main, and Hirsch (1926) recovered haemolysin from red cells sensitized by a particularly avid serum, by first washing with ether and then extracting with dilute acid The eluate contained 44×10^{-6} mg protein per haemolytic unit They considered that the preparation still contained protein derived from the antigen Euler and Brunius (1930) eluted haemolysin with glycine from sensitized stromata The eluate contained 84×10^{-6} mg of solid per haemolytic unit

Ottenberg and Stenbuck (1923–4) extracted agglutinin from sensitized typhoid bacilli with dilute alkali, and precipitated it three times with dilute solutions of cupric chloride at about pH 6 in an attempt to remove bacterial constituents The titre of the final product was 1/22 that of the original serum and contained 0 41 mg of nitrogen per 100 c c Protein could not be detected in it, although the same tests gave positive results with the original serum diluted to the same nitrogen content

[1] Schroeder (1930) found that blood-group agglutinins A and B dissolved in buffer solutions were not separated from protein by cataphoresis, but when dissolved in Ringer's solution the agglutinins went solely to the cathode, whereas the proteins moved towards both poles However, the technique of this experiment was highly unsatisfactory, a current of 30 milliamps was passed for 4 hours, it is probable that agglutinin at the anode was destroyed

Ottenberg and Stenbuck (1926) found that the isoelectric point of typhoid agglutinin extracted from sensitized typhoid bacilli by N/300 NaOH, but not further purified, was about pH 4 5, which differs from the points found by Michaelis and Davidsohn (1912) and Szent-Gyorgi (1921) This experiment of Ottenberg and Stenbuck is open to two serious objections The extract was not further purified and contained according to their previous experiments (1923–4) bacterial substance, it was therefore comparable to partially sensitized bacteria, whose isoelectric points will be discussed later Also citrate buffers were used in the cataphoresis experiments, Ito and Pauli (1929) have shown that such polyvalent anions shift the apparent isoelectric point of protein solutions to the acid side

The extracts prepared by Uchida (1927) were so weak in antibody that it is not surprising that no protein could be detected in them

Ramon (1923, a), by heating toxin-antitoxin floccules in diluted acid, recovered antitoxin solutions containing 0 012 mg of protein per unit Locke and Main (1926) obtained somewhat purer preparations As the figures of Hartley (1925, a, 1926) and Marrack and Smith (1930) show, this means that the antibody in the precipitates has been recovered together with all the protein As diphtheria toxin has been prepared (Locke and Main, 1928) containing only 0 0006 mg N per Lf unit, the antigen can only form a fraction of the total precipitate [1]

Recently Sumner and Kirk (1932) and Kirk and Sumner (1932) recovered antiurease from the urease-antiurease[2] precipitate by rubbing up with acid, which makes the urease insoluble The antiurease, which dissolved on neutralizing, was a globulin insoluble in distilled water and was completely precipitated on the addition of fresh antigen This preparation may have contained some antigen, as the urease is also a globulin which is precipitated from solution in distilled water on acidification with CO_2 (Sumner, 1926)

Probably the most satisfactory preparations are those obtained from the specific polysaccharides of pneumococci, as these can be prepared free from protein and only those of Type I, and Group IV, Type IV contain nitrogen Felton and Bailey (1926, b) proved that antibodies prepared by the methods used by Huntoon *et al* (1921) and Gay and Chickering (1915) contained some antigen Felton (1932) made use of the fact that the polysaccharides are precipitated by calcium and strontium hydroxide He dissolved the precipitates

[1] Pappenheimer and Robinson (1937) calculate that pure diphtheria toxin contains approximately 0 00046 mg N per Lf, a figure that agrees closely with the purest preparations of Eaton (1936) According to this calculation the antigen forms about 22 per cent of the specific precipitate at the neutral point However, in this calculation Pappenheimer and Robinson assume that no further protein is precipitated from the antitoxin solution on the addition of further amounts of antigen, after antibody ceases to be detectable in the supernatant fluid This is not the case with other antigen-antibody systems

[2] It is not necessary here to enter into the controversy whether Sumner's "crystalline urease" protein actually is the enzyme The essential point is that the antibody could be removed from a protein antigen

formed by antibody and the polysaccharide of Types I and II pneumococci with either of these hydroxides The active protein was precipitated on dialysis , redissolved, it contained 0 002 to 0 006 mg. of nitrogen to one protective unit, and was, therefore, of the same degree of purity with regard to protein as the best preparations obtained by Felton and Bailey (1926, b) in their repetition of Huntoon's (1921) work and as the preparations obtained by precipitation of the serum with $AlCl_3$ and $ZnCl_2$ Eighty to ninety per cent of the protein in these preparations was precipitated on addition of the specific polysaccharide [1] The more recent work on these lines, therefore, does not indicate that antibodies can be separated from proteins

B The Stability of Antibodies

1 *Heat*

There is a close correspondence between the destruction of antibodies and the denaturation of proteins by heat In many instances, though not invariably, the course is that of a mono-molecular reaction (Streng, 1909, Madsen and Streng, 1910), as found by Chick and Martin (1910) and Lewis (1926, a) in the heat denaturation of proteins (Fig XIII) The " Heats of Activation ",[2] calculated from the effects of increase of temperature on the rates of the two processes, are of the same order This value is very high, some ten times as high, for example, as that of the hydrolysis of sugar , the agreement therefore is of some value as evidence that the two processes, denaturation of protein and destruction of antibody, are the same [3] In the case of the agglutinins studies by Streng and Madsen and by Streng, it is possible that the apparent deviations from the course of a unimolecular reaction may have been due to the fact that more than one antibody (flagellar and somatic agglutinin) was involved in the reaction Yet, similar deviations were found by Gerlough and White (1934) working with tetanus antitoxin, which presumably contains but one antibody However, recent work, such as that of Heidelberger and Kendall (1935, b, c, d), has shown that the antibody molecules in a serum are not strictly uniform Deviations from the behaviour calculated for uniform molecules is, therefore, not surprising, and may well be much more striking in some sera than in others

Chick and Martin (1912) and Lewis (1926, b) found the rate of denaturation of proteins to be minimum at neutrality, that is, in the

[1] Similar preparations have been made by Heidelberger, Kendall and Teorell (1936), by dissolving the specific precipitate in strong salt solutions , also by Chow and Wu (1937)

[2] The Heat of Activation is the energy required to jolt a molecule out of a state of false equilibrium It may be compared to the energy required to lift a weight on to the parapet of a bridge in order to drop it over , it has no relation to the total energy change, or the free energy of a reaction

[3] From the data given it is possible to calculate the rate of heat destruction at lower temperatures It would take some thousand years to destroy as much antibody at 40° C as is destroyed in one hour at 70° C This does not agree with the results of Glenny (1913), so that it may be inferred that some other process was involved at this temperature

neighbourhood of pH 7 0 In accordance with this Gerlough and White (1934) found the rate of destruction of tetanus antitoxin at 60°C to be minimum at pH 6 8 and appreciably increased at pH 8 5 Streng (1909) found that heat destruction of agglutinins was much accelerated by the addition of alkali, even by an amount that would not raise the pH above about 9 0 Moloney and Taylor (1928) found increased destruction of diphtheria antitoxin at 55°C when the pH was reduced to 4 16 , even at room temperature some antitoxin was destroyed at pH 3 4 in 16 hours

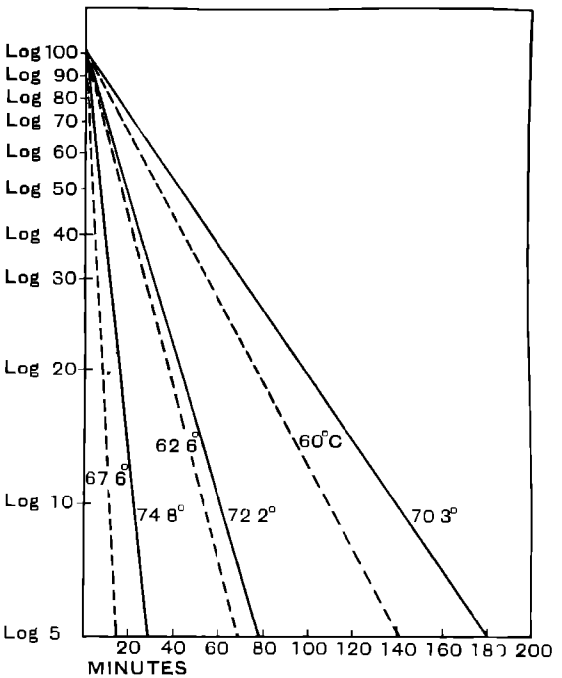

FIG XIII Rate of destruction of agglutinin (Streng, 1909) and denaturation of haemoglobin (Chick and Martin, 1910) at different temperatures Ordinates = logarithms of remaining antibody or undenatured protein

On the other hand, the heat denaturation of proteins is delayed by high concentrations of salts (Jordan-Lloyd, 1926) or of glycerol and, particularly, cane sugar (Beilinsson, 1929) Streng (1909) found destruction of agglutinins delayed by concentrations of sodium chloride above 2N , and Silber and Demidowa (1932) found agglutinins to be protected to a moderate degree by glycerol, and to a high degree by saturated cane sugar

However, the agreement between heat denaturation of proteins and antibody destruction is not complete Thus, the lability of antibodies

is very variable For example, Jones (1927, a) found that the flagellar agglutinin of the hog-cholera bacillus was not completely destroyed in 20 minutes even at 90° C , while the somatic agglutinin was completely destroyed at 75°C , and lost half its strength at 65°C in this time (Fig XIV) Streng (1909) found considerable differences in lability between different antibodies in the same sera this rules out extraneous factors, such as differences of pH He also found considerable differences between the agglutinins in the sera of different animals of the same species immunized with the same bacillus As, however, he did not distinguish between flagellar and somatic agglutinins, it is possible that, in these cases, he was dealing with agglutinins for two different antigens

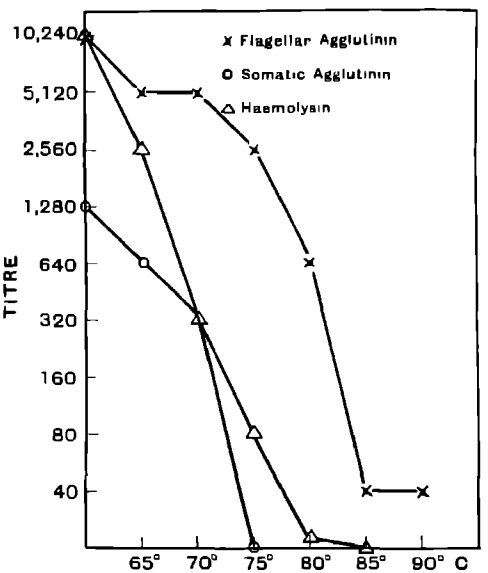

FIG XIV Degree of destruction of flagellar and somatic agglutinin for hog cholera bacillus, and of haemolysin, by heating for 20 minutes at temperatures from 65° to 90° C (Jones, 1927, a)

Also in some cases procedures which delay or prevent *coagulation only* of protein have some effect in reducing the rate of heat destruction of antibodies , for example, dilution with physiological saline (Streng, 1909) , bringing to high (Silber and Schaechter, 1927) or low pH (Koulikoff, Smirnoff, and Bobkova, 1928) , the addition of various substances such as urea and, particularly, a member of the polymethylene series, of high molecular weight, called fermentoid (Silber and Schaechter, 1927 , Silber and Nikolskaya, 1929) [1] It therefore

[1] The fact that destruction of antibody does take place under these circumstances, although no precipitation of protein occurs, disposes of the suggestion that antibody is merely carried down with the precipitated protein

appears that the destruction of some antibodies must be associated with the earliest degrees of heat denaturation of proteins, while others can resist even complete denaturation and are only put out of action completely by the coagulation and precipitation of the proteins Since coagulation is a polymolecular process this may account to some extent for deviation from the course of a monomolecular reaction sometimes found, particularly in the later stages, when coagulation is slow owing to the greater amount being already coagulated [1]

In most of the work on heat denaturation little or no precaution appears to have been taken to fix the pH of the antibody Different antibody solutions must vary in pH owing to differences of age and of methods of preparation , also a varying loss of CO_2, with consequent increase of pH, must occur during heating Such variations of pH may account for some deviations of the reaction from a regular course—compare the effect of changes of pH on the denaturation of egg albumin (Chick and Martin, 1912)—but will not wholly account for the differences in the lability of different antibodies

Chow, Lee and Wu (1937) incubated purified antibody to Type II pneumococcus with HCl (pH ca 2 5) at 37° As the protein became insoluble the antibody disappeared

2 *Alcohol*

The destruction of antibodies by alcohol runs parallel with the denaturation of proteins Mellanby (1908) found that above 4°C , the critical temperature, diphtheria antitoxin was destroyed at the same rate as the protein was denatured Merrill and Fleisher (1932) found that, if the concentration of alcohol was brought rapidly to over 90 per cent , protein was not denatured and agglutinin was not destroyed The denaturation of protein by alcohol is accelerated by acid or alkali (Wu, 1927) , Moloney and Taylor (1928) found that diphtheria antitoxin was more rapidly destroyed by small concentrations of alcohol when the pH was above 11 or below 4

3 *Organic Solvents*

Hartley (1925, b) extracted with ether antibodies which had been precipitated with alcohol in the cold He found that the power of combining with antigens was unaffected, although the power of flocculation might be reduced Further work on this line is discussed in Ch VI Merrill and Fleisher (1932) extracted agglutinins, precipitated by alcohol, with various organic solvents The titre of the agglutinin was not affected unless the solubility of the protein was reduced Glycerol dissolved the protein, which could then be reprecipitated with alcohol, the protein was not denatured by this process nor was the potency of the agglutinin reduced

[1] The rate of reaction of a polymolecular process diminishes to greater degree with time than does that of a monomolecular process

4 *Substitution in the Serum Proteins*

According to Reiner (1930) and Bronfenbrenner, Hettler and Eagle (1931), the introduction of substituents into the proteins of horse antisera by the azo-link (see next chapter) has little effect on the antibody activity, despite the complete loss of species specificity of the serum proteins Breinl and Haurowitz (1932), however, found that the titre of the bacterial agglutinins was reduced by this treatment Further work by Eagle, Smith and Vickers (1936) showed that the antibodies lost all reactivity on sufficient coupling of the proteins of the serum with diazo compounds However, the susceptibility of different antibodies to this treatment varied widely Also the different antibody activities of a single serum (for example, flocculating and protecting power of diphtheria antitoxin) were affected to a very different degree by the same treatment (a point which will be considered further in Chapter V Unitarian Hypothesis)

Breinl and Haurowitz (1932) also found that treatment with iodine or formaldehyde reduced the titre of agglutinins The effect of formaldehyde on antibodies has also been studied by Eisler and Lowenstein (1912), Baivy (1926), Braun (1933), Mudd and Joffe (1933) and Chow and Goebel (1935) All these workers agree in finding that the antibody was reduced by this treatment, although again different antibodies, and the different antibody activities of the same sera, were differently affected

The compound formed by the action of formaldehyde on amino groups is stable only when the pH is above 7 Chow and Goebel (1935) found that the precipitin activity of Type I anti-pneumococcal serum, which had been treated with formaldehyde, could be partially restored by adjusting the pH to 4 0 and keeping in the cold for several days

Chow and Goebel (1935) also acetylated the proteins of anti-pneumococcal antibody preparations (Type I) by treatment with ketene gas By this method one of the hydrogen atoms of the $-NH_2$ groups is substituted by an acetyl radical, and the proteins are not denatured The capacity of the preparations to form a precipitate with Type I polysaccharide was much reduced

These investigations agree in showing that chemical changes in the serum proteins reduce the activity of antibodies in a serum

5 *Enzymes*

Antibodies are destroyed rapidly by pepsin, less rapidly by trypsin (Pick, 1902 , Mellanby, 1908) Similar results were obtained by Schmidt and Tuljtschinskaya (1931–2) Huntoon *et al* (1921) cited as evidence of the non-protein nature of their purified pneumococcal antibody that it was not destroyed by trypsin Felton and Kaufman (1927), however, found that Huntoon's antibody, Types I and II antisera, and purified Types I, II, and III antibodies, were slowly destroyed by trypsin , as were also the antibodies highly purified by Al and Zn

precipitation (Felton, 1932) The antibody was more rapidly destroyed by pepsin, and by trypsin if phosphate buffers were added to fix the pH at 8 Breinl and Haurowitz (1930) have shown that, under the conditions employed by Huntoon, the digestion of globulin also is very slow A similar criticism would apply to the work of Frankel (1932), here it is claimed that "a considerable increase of free amino groups occurred", but no figures are given by which an estimate of the proportion of protein digested can be made When a purified precipitin, obtained from the precipitate formed by Type I pneumococcal polysaccharide and antiserum, was incubated with trypsin at pH 8, the destruction of precipitin ran parallel to the increase of amino nitrogen (Chow, Lee and Wu, 1937)

An important contribution to this subject has been made recently by Rosenheim (1937) She found that the flagellar (H) and somatic (O) agglutinins in the serum of horses and rabbits, in the earlier stages of immunization with *B typhosus*, were destroyed by pepsin, trypsin, and activated papain However, after several immunizing injections the H agglutinins became resistant to destruction by pepsin and trypsin (but not by activated papain) The free amino-N of solutions of globulin from such immune sera might be increased by 16 7 per cent of the total N in peptic digestion, and by 11 per cent in tryptic digestion without detectable loss of H agglutinin It does not follow, however, that all the globulin molecules were digested to this extent The antibody molecules themselves may have been resistant to dissociation by enzymes, or the antibody functions may have survived this degree of dissociation It cannot be inferred, from the demonstration of this enzyme-resistant antibody, that antibody is not a protein, it is hardly likely that antibody would be a protein in the early stages of immunization and cease to be a protein in the later stages

It is significant that this resistance to the action of pepsin and trypsin is shown by a flagellar agglutinin, since agglutinins have also proved particularly resistant to other treatment as by formaldehyde and by diazo compounds, and the flagellar agglutinin is more resistant to heat than is the somatic agglutinin The suggestion that the antibody function may be resistant to a certain degree of digestion is supported by the work of Pappenheimer and Robinson (1937) on diphtheria antitoxin prepared by digestion of serum with pepsin, under special conditions, according to the Parventjev process Quantitative investigations on the lines discussed in section C 2, indicate that the antibody molecules in these preparations are smaller than those in the normal sera It appears, therefore, that the part of the protein that carries the antibody function is more resistant to the action of enzymes than the rest of the molecule and than ordinary serum proteins Since antisera to heteroalbumoses, prepared by digestion with pepsin, will form precipitates with the parent protein (Landsteiner and van der Scheer, 1930–1931) some structural features of the protein must survive early stages of digestion The surviving antibody function may be attached to this remaining structure

6 *Specific Sera*

Landsteiner and Prasek (1911) found that agglutinins reacted like proteins with precipitins, that is, if precipitins for the serum of a given animal were added to such a serum containing agglutinins the agglutinins were removed The antibody was not merely "carried down" with the precipitate, for the formation of a precipitate by adding some foreign protein and its precipitin did not remove the agglutinin Similarly Eisler (1920) found that the antitoxin of antitetanus horse serum was removed by a precipitin to horse serum Moloney and Weld (1925) claimed that the titre of diphtheria antitoxin serum (horse) was unaltered by precipitation with anti-horse precipitin Smith and Marrack (1930, a), however, showed that the antitoxin was removed from the serum by precipitin, also that diphtheria toxin was bound by the precipitate so formed, and not by the precipitate from normal horse serum and precipitin

It is probable that the failures to demonstrate removal of antibodies from sera by precipitins is due to the use of amounts of precipitin unsuitable for maximum precipitation

C The Composition of the Antigen-Antibody Complex

It appears that if certain unconfirmed claims are excluded, attempts to prepare antibodies free from proteins have failed, and effects of various manipulations on antibodies are very similar to those on proteins

1 *Qualitative*

Further evidence of the protein nature of antibodies is derived from the composition of the antigen-antibody complex With precipitin reactions it was first shown that the precipitate contained considerably more protein than the antigen could account for (Welsh and Chapman, 1906, Dean and Webb, 1926) This became even more conclusive when the precipitin reaction with the non-protein specific carbohydrates was studied, since the antigen contained no protein Felton and Bailey (1926, c) found 2 5 mg of pneumococcal polysaccharide Type II precipitated 37 mg of protein, and that, like the purified antibody, the precipitate was least soluble at pH 6 6–7 Marrack and Smith (1930 and 1931, a) have shown that the ultraviolet absorption and other properties of diphtheria toxin-antitoxin floccules and the precipitate in a precipitin reaction were similar to those of serum globulin Analyses of the precipitate obtained with various antigens (see Section D) indicate that the antigen forms a minor part of these precipitates and that the major part is a protein resembling serum globulin that must be derived from the antiserum

It has been shown by numerous workers (see Eagle, 1930, b, 1935 and McCutcheon *et al*, 1930, for summaries) that particulate antigens such as bacteria, red blood cells and lipoid particles, when fully sensitized, move in an electric field as though they were coated with a layer of globulin (e g Shibley, 1926, Fig XV) Various workers have shown that the

isoelectric point of such antigens was shifted towards the neighbourhood of that of serum globulin by soaking in immune serum, the degree to which the point was shifted depending on the degree of sensitization The position of the isoelectric point was determined both by cataphoresis and by the optimum flocculation with salts The results of cataphoresis generally agree, although some of the work is rendered questionable by various technical errors, such as the employment of agar bridges, buffer solutions containing polyvalent ions, or too strong salt solutions with risks of heating The isoelectric points, so found, may be somewhat on the alkaline side of the isoelectric points of normal serum globulin (e g Joffe, 1935). But,

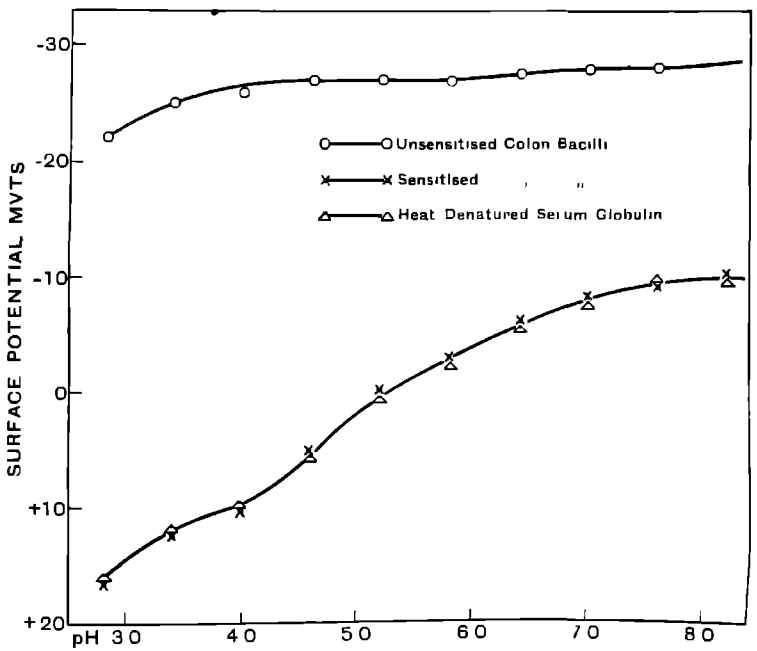

FIG XV Effect of pH on the surface potential of unsensitized colon bacilli, sensitized colon bacilli, and heat-denatured serum globulin (Shibley, 1926)

as noted later in this chapter, the isolated globulins which are considered to be the actual antibodies, may have similar abnormally alkaline isoelectric points When the proteins of an agglutinating serum are subjected to some treatment which alters the isoelectric points of the proteins without wholly destroying the antibody functions of the serum, the isoelectric point of the bacteria sensitized by the serum is also shifted Thus Mudd and Joffe (1933) found that, when agglutinating sera for *B pullorum* and for *B typhosus* were treated with formaldehyde, the isoelectric points of bacteria sensitized with these sera were shifted from pH 5 0 to pH 4 2 to 4 4

Similarly, treatment of agglutinating sera with diazo-benzene-sulphonic acid shifted the isoelectric points of sensitized bacteria to pH 3 35 (Eagle, Smith and Vickers, 1936) It may be inferred that these particulate antigens receive, on sensitization, a coat of substance that has an ionization curve and isoelectric point similar to those of serum globulin and is affected in the same way by chemical treatment Normal sera and other solutions of proteins also form surface layers on particles, in fact, particles so coated may be used for the study of the behaviour of proteins in an electric field (e g Abramson, 1932, Howitt and Prideaux, 1932) But considerably higher concentrations of normal than of immune sera are required to produce the effect on particulate antigens (cf Fig XXI), and the surface layers formed by normal sera are more easily removed by washing

Mudd and Mudd (1927) showed that whereas untreated acid-fast bacteria passed readily through a water-oil interface into the oil, sensitized bacteria remained in the water phase This showed that the surface layer of the bacteria was changed from one having greater attraction for oil than for water to one having a greater attraction for water than oil, a change which would be produced by covering the lipoidal surface of the bacteria by protein Here again the degree of surface changes varied with the degree of sensitization, and was much greater than that produced by soaking in normal sera

The protein attached to the antigens has been shown immunologically to be the same as the ordinary proteins of the antiserum Braun (1909) found that bacteria agglutinated with an agglutinating horse serum, when injected into guinea-pigs, sensitized them to a later injection of horse serum, and Altmann (1912) that rabbit red corpuscles treated with sheep immune serum produced precipitating and complement-fixing antibodies against sheep serum when injected into rabbits Similarly Smith and Marrack (1930, a) found that the floccules from diphtheria toxin and horse antitoxin, when resuspended, were flocculated by antisera to horse globulin

2 *Quantitative*

All the evidence, therefore, shows that when antigens combine with antibodies they take up an appreciable amount of globulin from the antiserum This globulin may be the actual antibody, or may be adsorbed non-specifically by the antigen-antibody compound Quantitative considerations favour the first supposition The purified preparations of pneumococcal antibodies consist mainly of protein Suppose the antibody is not protein, and is present in an amount as high as 10 per cent of the solids in such a solution as B III in the experiments of Heidelberger and Kendall (1929, c), there will then be present in 5 c c of the antibody solution 3 5 mg of this antibody This on the addition of 0 6 mg of specific polysaccharide gives a precipitate of 15 88 mg On the adsorption theory 4 1 mg of specific precipitate adsorb non-specifically 11 8 mg of protein from 10 c c of a 0 355 per cent solution of protein Comparing with this

the 170 mg of $Al(OH)_3$ required to adsorb the protein from 10 c c of a 1 per cent solution of protein (Spiegel-Adolf, 1932) we see that it implies an extraordinary adsorptive power on the part of the antibody-antigen compound. And this adsorption must be specific, for Heidelberger and Landsteiner (1923), Marrack and Smith (1931, b), and Haurowitz and Breinl (1933) have shown that coloured proteins that can be recognized are not carried down in the precipitate when unrelated antigens are precipitated by their antisera

Marrack and Smith (Marrack and Smith, 1930, 1931, a and b, Smith and Marrack, 1930, a) have studied the effect of the addition of large amounts of non-specific protein on the total amount of precipitate formed from a given mixture of antigen and antibody, and on its composition They found that the ratio of antigen to total protein in the precipitate depended only on the ratio in which antigen and antibody were mixed and was unaffected by the addition of non-specific protein, although in some cases the total amount of precipitate was increased (Table VI) The total antigen and antibody in a mixture are not always completely precipitated, even at optimum proportions, when antigen is in considerable excess this incomplete

TABLE VI

Effect of non-specific protein on amount and composition of specific precipitates (Smith and Marrack, 1930, a, Marrack and Smith, 1931, b)

	Protein in reacting mixture, per cent	*Mg N in precipitate per unit of antitoxin*	
Diphtheria toxin and purified antitoxin balanced mixture	0 033 1 22	0 00161 0 00163	Normal horse serum added
	Protein in supernatant fluid, per cent	*Mg N in precipitate from 1 c c of antiserum*	*Ratio, Antigen Total Protein in precipitate*	
Azo-protein and precipitin balanced mixture	1 8 5 45	0 282 0 287	15 4 15 6	Saline added Normal rabbit serum added
Azo-protein and precipitin antigen excess	1 9 5 6	0 320 0 445	20 8 20 1	Saline added Normal rabbit serum added

precipitation is usual Marrack and Smith consider that, since the amount of protein associated with a given amount of antigen in the precipitate was unaffected by the addition of non-specific protein to the reacting mixture, any increase in the total precipitate was due

to more complete precipitation and not to appreciable adsorption of the non-specific protein As would be expected, this increased precipitation occurred particularly when antigen was in excess Similarly, the ratio of nitrogen to polysaccharide in a precipitate is of the same order, whether the precipitate is formed by the addition of specific polysaccharide (Type III) to antipneumococcal serum, in which the specifically precipitable protein forms about 15 per cent of the total protein, or to an antibody solution in which the specifically precipitable protein is 50 to 60 per cent of the total (Heidelberger and Kendall, 1935, a) Heidelberger and Kendall (1935, a) have also found that the addition of normal horse serum to a purified solution of antibody to Type I pneumococcus did not increase the amount of protein precipitable by a given amount of Type I polysaccharide

Even more striking evidence is found in the work of Heidelberger and Kabat (1934, 1936) on the amount of nitrogen carried down from antisera in precipitin and agglutin reactions They found that the amount of nitrogen precipitated from an antipneumococcal serum (freed from antibodies to non-type-specific polysaccharide and proteins) by the type-specific polysaccharide was the same as the amount carried down by pneumococci of the same type when agglutinated If nitrogen were adsorbed non-specifically, it would be extremely unlikely that the amount adsorbed would be the same under such diverse conditions

The precipitate formed in a balanced mixture of diphtheria toxin and antitoxin, prepared by peptic digestion according to the Parfentjev process, contained $\frac{1}{6}$ the amount of nitrogen found in precipitates from balanced mixtures of toxin and undigested antitoxin (Pappenheimer and Robinson, 1937) This would be expected if the protein precipitated from the antitoxic serum is the actual antibody, and part of the antibody molecule is split off in the digestion process

A different line of approach has been used by Dean, Taylor and Adair (1935) They immunized rabbits both with egg albumin and horse serum albumin and found that the same amount of protein was precipitated by serum albumin from untreated serum or from serum from which a precipitate previously formed by the addition of egg albumin had been removed Similarly the amount of protein precipitated by egg albumin was not affected by a previous precipitation with serum albumin , and the amount of protein precipitated by egg albumin and serum albumin together was equal to the sum of the amounts precipitated by egg albumin and serum albumin separately

We may therefore say that when an antigen reacts with an antibody, a *certain definite amount* of the globulin of the antiserum, and not of other proteins, becomes bound to the antigen and is more or less completely precipitated It is natural to infer from this and the preceding evidence concerning the protein nature of antibody that this globulin which is bound by the antigen is the actual antibody , and that immune sera contain a certain definite amount of globulin

which is so altered that it will react with the appropriate antigen This opinion is supported by the work of Heidelberger, Sia, and Kendall (1930), in which they showed that the titre of an anti-pneumococcus serum was roughly proportional to the amount of protein precipitable from it by excess of specific carbohydrate But a serious objection is found in the work of Taylor (1931), which appeared to show, from the amount of antibody found in the supernatant fluid, that the amount of antibody (rabbit anti-horse serum) precipitated by a given amount of antigen (horse serum) was not increased when antibody was added in excess of optimum proportions, although Dean and Webb (1926) had shown that the amount of protein carried down from the antiserum by a given amount of antigen was increased under these conditions The optimum proportions method is sufficiently accurate to detect the removal of an amount of antibody equivalent to the increased protein in the precipitate, when the antibody excess is not great It can only be suggested that the residual antibody is in some way modified, so that the optimum proportion method no longer gives an accurate measure of its amount There is considerable evidence that the antibody molecules in a given serum are not uniform When part of the antibody is precipitated by antigen, the antibody molecules left in solution are probably those of lower affinity for antigen and may well differ in their behaviour in the optimum proportions reaction Certainly the precipitates obtained, when antigen is mixed with antibody in excess of optimum proportions, contain excess of antibody in some cases Thus, diphtheria-toxin-antitoxin floccules are themselves antitoxic, when precipitated from a mixture containing antitoxin in excess (Ramon, 1922) Marrack and Smith (1931, b) give additional evidence that, in a precipitin reaction with azo-protein as antigen, the increased ratio of protein to antigen in the precipitate, obtained when antibody is added in excess, corresponds to an increase of the amount of antibody precipitated by a given amount of antigen Another serious difficulty has been brought out by quantitative studies of precipitin reactions The amount of protein precipitated from an antiserum on the addition of antigen does not reach its maximum at the equilibrium point, that is, the point where neither antigen *nor antibody* is detectable in the supernatant fluid The maximum is reached when the excess of antigen has been added (Ch VI, Section M) It appears as though some protein was precipitated, when excess of antigen was added, that did not function as antibody The work of Heidelberger and Kendall (1935, b, c, d) suggests an explanation The antibody molecules are not uniform, some have such a weak affinity for antigen, or so few combining groups, that they can be built into a precipitate with antigen only when more "avid" antibody molecules are present In the absence of such more avid molecules they cannot form a precipitate with antigen When antigen is not in excess it combines mainly with the more avid molecules and leaves the less avid free At the first point at which neither antigen nor

antibody can be detected in the supernatant fluid after a precipitate has formed some of these " non-avid " antibody molecules are left in solution Only an excess of antigen precipitates all the antibody

The objection might be raised to this explanation that it amounts merely to calling the non-specific protein, which is precipitated by excess antigen, " non-avid " antibody This objection is met by the fact that the total amount of protein precipitable from an antiserum by antigen is constant and is not increased by the addition of normal serum

D The Relation of Antibody Globulin to Normal Globulin

It appears, therefore that the properties of antibodies indicate that they consist of modified serum globulin The question then arises wherein the difference between normal and antibody globulin lies, and whether any differences, other than the specific power of combining with antigens, can be demonstrated The antibody molecules may differ either in their general structure or in the presence of a prosthetic group

As pointed out in Chapter I there is a possibility that the fractions prepared from serum globulin are artefacts The confinement of some antibodies to definite fractions (e g diphtheria antitoxin and pneumococcal antibodies of horse serum) suggests that the molecules from which these fractions are formed differ from the remaining globulin molecules But the great variability in the behaviour of antibodies to the same antigen on fractionation, the appearance of an antibody in one fraction when prepared from the serum of one animal and in another fraction when prepared from the serum of another animal, show that in this respect there is no evidence of an essential peculiarity, *qua* antibody to a particular antigen, in antibody globulins

Exception might be made of the pneumococcal antibodies of horse serum which do constantly appear in and form the greater part of certain globulin fractions Felton (1926), comparing antipneumococcal sera with normal sera, found a larger proportion of the globulin of the antipneumococcal sera precipitated, on dilution, in the neighbourhood of pH 6 8 And as the fraction precipitated at this pH was mainly composed of antibody globulin, he considered that the antibody globulin formed a globulin fraction not present in normal serum

Reiner and Reiner (1932) showed that a fraction with similar properties can be separated from normal horse serum It might be inferred that the isoelectric point of these globulin fractions was in an abnormally alkaline range Preparations of Type I antibody were made by a special method elaborated by Chow and Goebel (1935), the greater part of the protein in this preparation was precipitable by acetylated Type I polysaccharide In the presence of low concentrations of electrolytes the protein in these preparations was insoluble at pH 7 6 and dissolved on the addition of small amounts of acid or alkali Chow and Goebel inferred that the

isoelectric points of these antibodies lay near pH 7 6, and anticipated that these proteins would contain an abnormally high proportion of basic amino-acids This was not the case, the lysine was only slightly above that of normal serum globulin However, Heidelberger, Pedersen and Tiselius (1936), using actual cataphoresis methods, found the isoelectric point of antibody to Type I pneumococcus (from horse serum, recovered from the specific precipitate) to be at pH 4 8 The isoelectric point of preparations from anti-pneumococcal horse sera by Felton's method lay between pH 5 0 and 5 2, while that of the preparation from normal horse sera by this method was at pH 5 7 The isoelectric point of the antibody recovered from the specific precipitate formed by rabbit antisera to Type III pneumococcus and Type III polysaccharide, was at pH 6 6, but the protein did not appear homogeneous

Ultracentrifuge methods have shown that the fractions prepared by Felton's method from normal and antipneumococcal horse serum differ considerably in particle size (Table VII) The antibodies to pneumococci in horse serum appear to be characterized by a sedimentation constant[1] (and consequently particle size) considerably higher than that of the chief fraction of the globulin of normal serum, although a small fraction with this high sedimentation constant is also present in normal serum

Preparations of the antibody can be obtained in which the protein is solely in the form of these particles and removal of antibody from serum by the specific polysaccharide reduces the number of large molecules in the serum

However, in the antibody preparations recovered from the specific precipitate by extraction with $Ba(OH)_2$ the large particles are much broken up, without loss of the ability to precipitate with the polysaccharide Also rabbit pneumococcal antibodies do not differ from normal serum globulin in particle size and cannot be precipitated from serum by dilution with distilled water (Heidelberger and Kendall, 1933) Neither the peculiar solubility nor the large particle size can be considered essential characteristics of pneumococcal antibodies

If the claims to have raised the concentration factor of antibodies appreciably by adsorption and elution are correct, these must be based on differences from normal globulins An indication of similar differences is given by the work of Reitstotter (1920), Freundlich and Beck (1925), and Rabinerson (1926) Reitstotter found that ferric hydroxide sol was precipitated at lower salt concentrations when mixed with pseudoglobulin from antisera than when mixed with pseudoglobulin from normal sera Freundlich and Beck confirmed this, the differences were not very striking, but appeared constant

[1] Sedimentation constants are given rather than molecular weights, as the sedimentation constant depends on the shape of particles as well as on their weight Further data are required in order to calculate the molecular weights of these antibodies, but it can be said that they are three or four times as heavy as the molecules of normal serum globulin

TABLE VII

Sedimentation Constants and Isoelectric Points of Antibodies

Antibody Preparation	*Method of Preparation*	*Sedimentation Constant*	*Isoelectric Point pH*
Anti-Pneumococcal, Types I, II and VIII (horse serum)	Felton	Principal Portion, 15×10^{-13} Not homogeneous (A)	
Anti-Pneumococcal, Type I (horse serum)	,,	Principal Portion, 18×10^{-13} Large Portion, $6\ 8 \times 10^{-13}$ Small Portion, 24×10^{-13} (B)	5 0 to 5 2 (C)
Normal horse serum	,	9×10^{-13} and 17×10^{-13} (A)	
,, ,, ,,		Principal Portion, $8\ 1 \times 10^{-13}$ not homogeneous Small Portion, $12\ 9 \times 10^{-13}$ and $18\ 5 \times 10^{-13}$ (B)	5 7 Rather inhomogeneous (C)
Normal horse serum globulin	Ammonium sulphate precipitation	$7\ 1 \times 10^{-13}$	5 2, also more alkaline fractions (D)
Anti-Pneumococcal, Type I (horse serum)	Dissociated from specific precipitate	$18\ 2 \times 10^{-13}$ Almost homogeneous (B)	4 8, homogeneous (C)
Anti-Pneumococcal Type III (rabbit serum)	, ,,	$7\ 0 \times 10^{-13}$ Also minor fractions (B)	6 6, not homogeneous (C)
Normal rabbit serum globulin	Sodium sulphate precipitation	Principal Portion, $7\ 1 \times 10^{-13}$ Small Portion $18\ 4 \times 10^{-13}$ Also minor fractions (B)	
Anti-egg-albumin serum globulin (Rabbit)	,, ,,	Principal Portion, $7\ 4 \times 10^{-13}$ Small Portion, $19\ 6 \times 10^{-13}$ (B)	
Anti-azo-protein serum (Rabbit)	Untreated	7×10^{-13} (A)	
Haemolysin for sheeps' red blood corpuscles	Untreated serum		About 6 3 Positive at 5 25 and 5 6 (E)
Agglutinin for *B paratyphosus B*	,,		,, ,, (E)
The greater part of the protein of haemolytic and agglutinating sera			Negative at 5 2 to 8 2

(A) Biscoe, Hercik and Wyckoff (1936)
(B) Heidelberger and Pedersen (1937)
(C) Heidelberger, Pedersen and Tiselius (1936)
(D) Tiselius (1937)
(E) Girard and Louranc (1934).

Rabinerson obtained similar results with globulin from diphtheria antitoxin However, Mutzenbecher (1931) found that the water-soluble globulin, obtained from diphtheria antitoxic sera by electrodialysis behaved in the opposite way, and that the effect of pseudoglobulin prepared by the method used by Reitstotter was within the limits of the effects produced by the preparations from normal sera

Kasarnowsky (1929) found prussian blue sol to flocculate at a different pH when mixed with diphtheria antitoxin serum to that at which it flocculated when mixed with normal serum The pseudoglobulin concentration in the two sera was said to be the same It is very possible that differences of preparation, &c , may have a considerable influence on these results The published experiments are not sufficiently numerous or consistent to show that the differences between antibody globulin and normal globulin are invariable Also in most antisera the antibody globulin forms only a small part of the total protein It is unlikely, therefore, that any differences in composition of antibody and normal globulin would be detectable except in highly purified preparations

The nitrogen content of purified pneumococcal antibodies (Felton, 1932) agrees well with that found for normal globulin (Adair and Robinson, 1930) Pseudoglobulin from diphtheria antitoxin serum may contain a high percentage of antibody globulin , Smith, Brown, and Gross (1932) found that the nitrogen content of such pseudoglobulin was the same as that of pseudoglobulin from normal serum Analyses of pseudoglobulin fractions of diphtheria and tetanus antitoxin sera did not differ significantly from those obtained for normal sera (Banzhaf, Sugiura, and Falk, 1917) Analyses of various specially purified antibody preparations and of antigen-antibody precipitates are given in Table VIII As a whole, the differences found between the different samples are not greater than might be expected from the use of different methods by different workers The amounts of histidine found by Calvery and his colleagues in Type I and II floccules are very low compared with the amounts found by Chow and Goebel in the purified antibody This may be due to the difference of methods , nitrogen of other amino-acids may be included in the histidine nitrogen of the Van Slyke partition method (see Calvery, 1929)

As already noted, Rosenheim (1937) showed a progressive resistance of agglutinins to destruction by pepsin and trypsin as immunization was prolonged This resistance might be interpreted in various ways The antibody globulin as a whole might be supposed to be abnormally resistant to digestion, or the antibody function of the antibody molecule might be supposed to survive a certain degree of digestion Rosenheim suggested an explanation along the second line —that the formation of binding sites for antigen may occur in the terminal or exposed amino-acids in the globulin molecule in the early stages of immunization, and in more central parts of the molecule in later stages Removal of terminal amino-acids by pepsin and trypsin would remove the active groups of the first type of

TABLE VIII

Composition of purified antibody preparations and of antigen-antibody precipitates

	Normal Horse Globulin (A) Mean	*Floccules Diphtheria Toxin Antitoxin (A)*	*Normal Horse Globulin (B)*	*Purified Type I Pneumococcal Antibody (B)*	*Floccules Type II Pneumococcal Polysaccharide and Antibody (C)*	*Floccules Type I Pneumococcal Polysaccharide and Antibody (D)*	*Floccules Egg Albumin and Antibody (E)*	*Normal Horse Globulin (F)*	*Normal Ox Globulin (F)*	*Floccules Haemoglobin and*	
										Antiserum from Horse (F)	*Antiserum from Ox (F)*
Amide N	8 9	9 1	9 46	9 22	3 6	4 5				4 3	
Mon-amino N	66 9	67 8			75	75				70 0	
Di-amino N	24 3	22 9								21	
Cystine N	[2 76] F and M	[2 05] F and M	1 09	1 25	[3 1] F and M	[2 55] F and M		[1 5] F and L	[2 1] F and L	[1 8] F and L	[1 8] F and L
Tyrosine	[6 53] F and M	[5 85] F and M			[5 5] F and M	[5 5] F and M		[3 8] F and L	[3 3] F and L	[4 0] F and L	[3 3] F and L
Tryptophan	[1 78] F and M	[1 80] F and M			[2 0] F and M	[2 0] F and M					
Arginine N			8 98	8 00	[5 5]	[4 95]		[4 8]	[5 0]	[5 8]	[5 0]
Histidine N			5 85	5 98	[1 1]	[0 95]		[3 4]	[4 0]	[4 4]	[4 4]
Lysine N			8 31	9 97	[4 8]	[5 35]					
Nitrogen	[15 13] G			[14 85] (2 75 per cent of lipoid) [14 45] H	[16 0]	[15 85]	[15 0 to 15 4]			[17 7]	

A Hewitt (1934)
B Chow and Goebel (1935)
C Calvery, Heidelberger and Kendall (1935)
D Calvery (1935)
E Taylor, Adair and Adair (1934)
F Breinl and Haurowitz (1930)
G Adair and Robinson (1930)
H Chow, Lee and Wu (1937)
F and L Folin and Looney (1921) method
F and M Folin and Marenzi (1929) method

Figures in brackets are the percentage of nitrogen or of amino-acid in the substance analysed
Figures not in brackets are the percentage distribution of nitrogen

antibody, but expose fresh active groups of the second type Since papain attacks any peptide link in the protein molecule, it will break up active or potentially active groups whether terminal or not, and destroy the agglutinating power of both types of antibody This explanation involves assumptions concerning the structure of proteins and the action of proteolytic enzymes which may not be correct (in Wrinch's cyclol molecules there are no terminal amino-acids)

The quantitative work of Pappenheimer and Robinson (1937) indicates that the actual antibody molecule is not resistant to proteolytic enzymes, but that the antibody function survives a certain degree of digestion, owing either to the uncovering of fresh binding sites, as suggested by Rosenheim, or to the special resistance to digestion of part of the antibody molecules

The change in ultraviolet absorption found by Takodora and Nakayama (1920) is accounted for by increase of serum globulin, which usually occurs on immunization

There does not therefore seem to be any consistent chemical or physical difference between antibody globulin and normal globulin other than the possible resistance of part of the molecule to the action of proteolytic enzymes

The peculiarity of structure or prosthetic group, which converts a globulin molecule into an antibody, may be of very varying degrees of stability It may be destroyed by manipulations which would leave ordinary globulin unaffected, or survive a considerable alteration in the globulin molecule as a whole This would account for the great differences in the heat stability of different antibodies and for their survival of some degree of digestion by trypsin The power to act as an antibody must depend on some difference from normal globulin, and, so long as antibodies do not resist treatment that completely destroys the globulin, differences in stability cannot be regarded as evidence of the non-protein nature of antibodies

E The Increase of Serum Proteins on Immunization

On immunization there may be a considerable increase of serum globulin Investigations have been made mainly on the sera of horses immunized against diphtheria toxin Reymann's (1924) average figures for 24 horses show an increase of globulin from 4 17 to 6 27 per cent with the development of 325 units of antitoxin per c c The globulin fraction not precipitated by one-third saturation with ammonium sulphate was chiefly affected, rising from 3 28 to 5 15 per cent , the globulin fraction precipitated by one-third saturation rose, to a less degree, in the earlier stages of immunization, but the increase was not maintained Albumin was reduced from 3 01 to 2 29 per cent Similar results have been obtained by others (Hiss and Atkinson, 1900–1 , Gibson and Banzhaf, 1910 , Meyer, Hurwitz and Taussig, 1918 , Kirkbride and Murdick, 1927) Joachim (1903), however, found in one serum containing 500 units per c.c that the chief increase was of the fraction precipitated by one-third

saturation with ammonium sulphate Ledingham (1907) also found in one horse serum a considerable increase of this fraction, but the partition of the globulin between the two fractions was very variable The reduction of albumin does not always occur, and in such cases a great increase of total protein may be found, up to 15 per cent Ionesco-Mihaiesti and others (1935) found that the titre of antitoxic sera does not rise to high levels until the concentration of globulin begins to rise The increase of globulin does not run parallel to the antitoxic titre Thus, Kirkbride and Murdick (1927) found only 6 52 per cent of pseudoglobulin (6 89 per cent of total globulin) in a serum containing 2,050 antitoxic units The antitoxic titre may remain high although the globulin concentration falls (Ionesco-Mihaiesti and others, 1935) An increase of globulin also occurs in the serum of refractory horses (Reymann)

Similar but less extreme changes of proteins occur in the serum of horses immunized with typhoid bacilli, colon bacilli and cholera vibrios (Ionesco-Mihaiesti and others, 1935) Langstein and Mayer (1904) found both globulin fractions, that soluble and that insoluble in one-third saturated ammonium sulphate, increased in the serum of rabbits immunized with typhoid bacilli, pneumococci and streptococci, the albumin was reduced Hurwitz and Meyer (1916) found the increase of globulin very irregular on immunizing Belgian hares with typhoid bacilli, dysentery bacilli (Shiga), and staphylococci Hewitt (1934) found an increase in globulin in horse serum from 4 1 to 7 9 after immunization with a haemolytic streptococcus Immunization of rabbits with pneumococci causes an increase of the total globulin, but not of the globulin precipitated by 10 per cent methyl alcohol (euglobulin), a steep rise of total globulin precedes a steep rise of antibody, but the antibody and increase of total globulin run roughly parallel In horses immunization causes an increase of the globulin precipitated by 10 per cent methyl alcohol (Liu, Chow and Lee, 1937), but only a slight increase of the fraction, characterised by peculiar solubility, in which the actual antibody is found (Liu, Wu and Chow, 1937) Doerr and Berger (1921) and Berger (1922) found a very considerable increase in the serum globulin of rabbits immunized with foreign serum or red blood corpuscles Hartley (1914, b) found the globulin insoluble in saturated NaCl solution, increased in cattle hyperimmunized against rinderpest

Since only about 0 01 mg of globulin per unit of antitoxin is precipitable by the addition of diphtheria toxin, only a fraction of the usual increase of globulin can be regarded as the actual antitoxic globulin At most $\frac{2}{3}$, usually under $\frac{1}{2}$, of the increase of globulin in rabbit's serum, on immunization with pneumococci is accounted for by antibody (Liu, Chow and Lee, 1937) It appears that a considerable new formation of globulin occurs in response to the stimulus of the antigen, but that only a part of this can actually react with the antigen

An interesting instance of the connexion between serum globulin

and antibodies is seen in the development of antibodies in the serum of calves (Orcutt and Howe, 1922) At birth the serum contains no agglutinins for *Brucella abortus* and the amount of globulin precipitable by the lower concentrations of sodium sulphate is very small Colostrum contains agglutinins, and also much more of a globulin apparently identical with serum globulin than does ordinary milk ; after its ingestion the fraction of the globulin precipitated by low concentrations of sodium sulphate rises considerably in the calves' serum within a few hours (e g from 0 5 to 3 0 per cent) , at the same time agglutinins appear in the serum up to a titre of 1/100 to 1/12,000 Ordinary milk does not have this effect

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CHAPTER III

THE SPECIFICITY OF ANTIGENS[1]

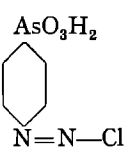
A Artificial Protein Antigens

1 *Azo-proteins*

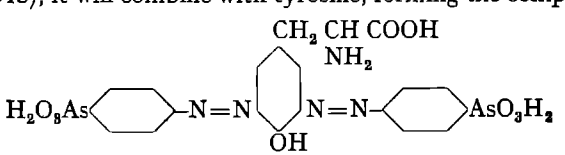
In discussion of the nature of the immunity reactions it is necessary to consider first the simplest reactions which involve the fewest unknown processes We shall therefore deal mainly with precipitin and agglutinin reactions Again, naturally occurring antigens do not present such variations as would make them suitable for the study of the effect of changes of chemical composition and configuration on their immunological character It is therefore necessary to make use of artificial preparations The most successful method is that introduced by Landsteiner and Lampl (1917, c) , it is best illustrated by an example

Atoxyl (*p*-amino-benzene-arsinic acid)

when mixed with sodium nitrite and HCl is diazotized and forms



this when mixed with an aromatic substance which has an — OH group in the benzene ring, or with various hetercyclic compounds, couples with them, forming azo-dyes In particular, as shown by Pauli (1915), it will combine with tyrosine, forming the compound

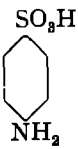


When the diazotized atoxyl is mixed with a protein in alkaline solution it combines, presumably with tyrosine and histidine of the protein, and forms a dye, red in alkaline and yellow in acid solution

[1] For a fuller discussion the reader is referred to " The Specificity of Serological Reactions," K Landsteiner (1936, a)

Such a compound Landsteiner called an atoxyl-azo-protein.[1] This treatment gives the protein a new immunological character dependent on the "determinant group", atoxyl, which has been attached. This new character may be shown in four ways

(1) The serum of a rabbit immunized with atoxyl-azo-protein will give a precipitate with any other protein coupled with diazotized atoxyl For example, horse-serum proteins coupled with diazotized atoxyl may be used for immunizing a rabbit, the antiserum produced will give a precipitate with chicken-serum proteins or egg albumin coupled with diazotized atoxyl[2] The reaction is specific, such a serum will not give a precipitate with protein coupled with diazotized sulphanilic acid



(2) If a guinea pig has been sensitized with one atoxyl-azo-protein, the injection of another atoxyl-azo-protein will produce anaphylactic shock (Landsteiner, 1924)

(3) The formation of a precipitate by atoxyl-azo-protein and antiserum is prevented by the addition of an excess of relatively simple substances containing atoxyl, such as diazotized atoxyl coupled to

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[1] Klopstock and Selter (1928) mixed diazotized atoxyl and proteins in "neutral" solution and found that the product (which they considered a mixture) behaved immunologically like the azo-compound This experiment is quoted as indicating the possibility that new immunological characters may be introduced into proteins by mere mixing with various substances, and as suggesting an explanation of drug idiosyncrasies It is, however, obvious at first sight, and has been conclusively demonstrated (Heidelberger and Kendall, 1929, a), that actual combination of protein and diazotized atoxyl occurs in neutral solution If enough of the diazo-compound is added combination may also occur with the indole group of tryptophan, the NH group of proline and oxyproline, the imidazole group of histidine and with free NH_2 groups

[2] The original immunological character of the protein may not be wholly destroyed That is, the serum in the example may still give a precipitate with untreated horse-serum proteins It is therefore necessary to test for the appearance and specificity of the new immunological character with a "test antigen", that is, an unrelated protein coupled to the "determinant group", which in this case is diazotized atoxyl

It is necessary to introduce a terminology in order to avoid repetition or vagueness Two antigens A and B will be said to be "equivalent" when the antiserum to A reacts in the same titre both with A and B, and vice versa A and B will be said to be "partially equivalent" or to give cross-reactions when the antiserum to A does not give such a strong reaction (in titre or amount, for example, of precipitate) with B as with A or vice versa A and B are said to be immunologically different when the antiserum to A gives no reaction with B and vice versa It should, however, be realized that in most cases the method of test of the equivalence of two antigens has been relatively rough Quantitative estimations of the amount of antibody precipitated may reveal differences between antigens that have been considered equivalent

tyrosine, by atoxyl itself in higher concentrations, and even by arsenic acid in sufficient amount (Landsteiner, 1920, Table IX) These simple substances are not antigenic, that is, they will not produce antisera when injected into animals Nor, as a general rule, will they give precipitates with antisera to the homologous azo-proteins, nor produce anaphylactic shock in guinea-pigs sensitized by the homologous azo-protein They will in the remainder of this work be called " simple haptenes "

TABLE IX

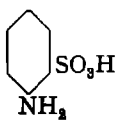
Effect of various compounds on the precipitate formation by antiserum to p-*amino-benzene-arsinic acid (atoxyl) diazotized and coupled to protein, and a test antigen*

Substance added	p-*amino benzene arsinic acid diazotized and coupled with tyrosine*			p *amino benzene arsinic acid*		*Benzene arsinic acid*	o *amino-benzene arsinic acid*	*Methyl arsinic acid*	p-*amino-benzene sulphonic acid*
Concentration used	M/10,800	M/1 200	M/400	M/270	M/90 M/30	M/70	M/70	M/70	M/70
Degree of precipitation	++	±	0	++	+ 0	0	0	++	++

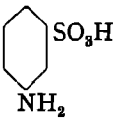
(4) A guinea pig sensitized with atoxyl-azo-protein can be protected against the production of anaphylactic shock by a discharging dose of another atoxyl-azo-protein by injecting, before the " discharging " dose, an excess of simple haptene (Landsteiner, 1924).

These last three reactions are also specific If the animal is immunized or sensitized with atoxyl-azo-protein, atoxyl is the essential constituent in the subsequent reactions, and another aromatic amine, such as sulphanilic acid cannot be used in its place The use of these azo-derivatives presents the advantages of the several methods by which the determination of immunological character can be studied The greatest advantage, however, lies in the variety of " determinant groups " that can be attached The substances attached to proteins in this way contain a benzene ring, an $-NH_2$ group must be attached to one of the carbon atoms of this ring in order to provide the $-N=N-$ link to the protein A great range of atoms or groups of atoms may be attached to the other five carbon atoms of this ring Such substituents occupy the distal position, are the parts of the azo-protein presented towards other molecules, and may in many cases wholly or mainly determine the immunological character

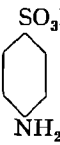
The first " determinant groups " thus employed were aromatic acids Landsteiner and Lampl (1918) tested the reaction of 35 antigens, so formed, with antisera to 23 antigens With the simple precipitin reaction the specificity was not absolute, it was determined partly by the acid radicle and partly by the position of this radicle in the benzene ring Thus proteins coupled with *o*-amino-benzene-sulphonic acid



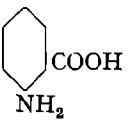
gave precipitates with antisera to both *o*-amino-benzene-sulphonic and *m*-amino-benzene-sulphonic



azo-proteins, but not with the antiserum to the *p*-derivative

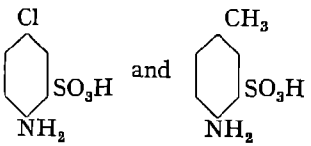


also with antisera to *o*-amino-benzoic

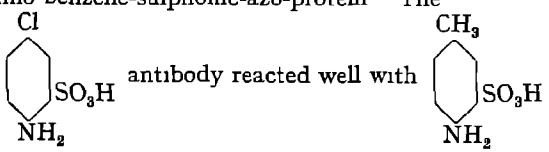


azo-protein, but not with the antisera to the *m*- and *p*-amino-benzoic-azo-proteins

The introduction of further substituents for —H in the benzene ring affected the specificity Thus, the antiserum to *o*-amino-benzene-sulphonic-azo-proteins did not react with the proteins coupled with

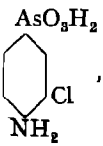


and the antisera to these two latter compounds reacted only slightly with *o*-amino-benzene-sulphonic-azo-protein The

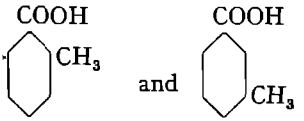


azo-protein and vice versa

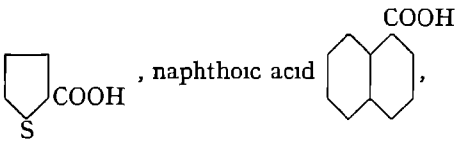
The inhibition reaction showed a rather less degree of specificity, Thus the reaction between *p*-amino-benzene-arsinic-acid antibody and the homologous antigen was inhibited not only by *p*-amino-benzene-arsinic acid but also by *o*-amino-benzene-arsinic acid and by 3-chloro-4-amino-benzene-arsinic acid



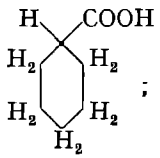
and that between *o*-amino-benzoic-acid antibody and the homologous antigen was inhibited (Landsteiner and van der Scheer, 1931, a) both by benzoic acid, and by benzoic acid substituted either in the *o*- or *m*-position with $-CH_3$, $-Cl$, $-Br$, or $-NO_2$, e g by both



It is interesting that the benzene ring is not indispensable for inhibition Thus the *p*-amino-benzene-arsinic acid (atoxyl) reaction was inhibited by arsenic acid, though hardly at all by methyl arsinic, $CH_3AsH_2O_3$, and the *o*-amino-benzoic acid reaction by other ring compounds, for example, thiophene carboxylic acid

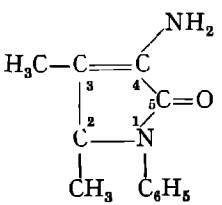


and *cyclo*-hexane-carboxylic acid



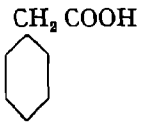
also to a less degree by fatty acids, the greatest effect being obtained with caproic, C_5H_{11} COOH, and heptylic, C_6H_{13} COOH, and less with acids of lower molecular weight (Landsteiner and van der Scheer, 1931, a).

Erlenmeyer and Berger (1934) show that in pyrazolon compounds specificity was affected by the position of a double bond Precipitate formation by antigens, formed from 1–phenyl–2, 3–dimethyl–4–amino–5–pyrazolon



diazotized and coupled with protein, and the corresponding antiserum was inhibited by other compounds with a double bond between the 3 and 4 C atoms, it was not inhibited by compounds with the double bond between the 2 and 3 C atoms

When the determinant groups in antigens are benzene ring derivatives with a carbon chain attached to the ring the specificity becomes less sharp as the number of carbon atoms in the chain is increased Thus while phenyl-acetic acid

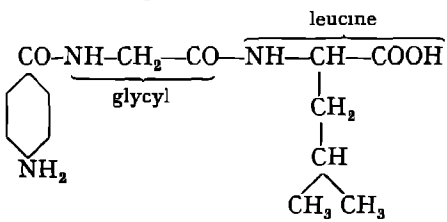


is immunologically distinct from benzoic acid the longer chain compounds phenyl-butyric acid

and phenyl-caproic acid

are almost equivalent (Landsteiner and van der Scheer, 1934, a)

A set of experiments which is of great interest in connexion with the specificity of proteins were made with peptides by Landsteiner and van der Scheer (1932) Compounds, of which *p*-amino-benzoyl-glycyl-leucine is an example,



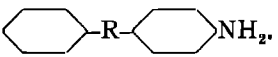
were synthesized and used as " determinant groups " Calling this compound G L (= glycyl-leucine) and the corresponding -glycyl-glycine, -leucyl-glycine, and -leucyl-leucine compounds G G , L G , and L L respectively, it was found that, for example, G L antiserum gave a precipitate with G L antigen, less with L L antigen, and less or none with G G and L G antigens The reaction of G L antiserum with G L antigen was inhibited by *p*-amino-benzoyl-glycyl-leucine and nearly as strongly by *p*-amino-benzoyl-leucyl-leucine, but only very slightly affected by the similar G G and L G compounds The specificity is determined, therefore, by the nature of both amino-acids, but the principal factor is the terminal amino-acid Further work by Landsteiner and van der Scheer (1934, b) with polypeptides containing up to five amino-acids confirms the predominant effect of the terminal acid and lesser effect of the nature, order and number of the non-terminal amino-acids Thus the reaction between the L G antigen and the homologous antiserum was inhibited by nitro-benzoyl-L G compound, but not by nitro-benzoyl-G L , L G G and G G L , and only slightly inhibited by nitro-benzoyl-G L.G

When the " determinant groups " do not contain acid radicles the specificity is less sharp (Landsteiner and van der Scheer, 1927) As already indicated, the position of substituents in the aromatic nucleus is of more importance than their nature Thus *p*-chloro-aniline

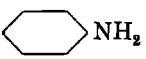
antibody reacted equally with *p*-nitro, *p*-chloro, *p*-bromo aniline and *p*-toluidine

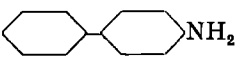
antigens

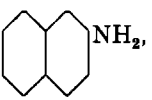
This work has been extended to include determinant groups that contain more than one benzene ring Erlenmeyer and Berger (1932) studied a series of compounds

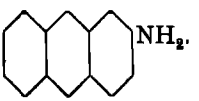


and found that those in which R represented CH_2, O or NH were equivalent and sharply distinguished from the CO compound However, Jacobs (1937) did not confirm this He found cross reactions between the CH_2, O and CO compounds, varying greatly with different sera (Table X) Cross reactions with the N = N compound were much weaker This may be in part due to the greater length of this bond and to the fact that the N = N compounds are probably trans compounds, while the other compounds are necessarily cis Jacobs also found but weak cross reactions between this group of compounds,

aniline 

p-amino-diphenyl 

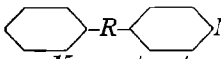
β-naphthylamine 

and *β*-anthramine 

Similar results were obtained by Mutsaars and Grégoire (1936) with aniline and naphthylamine The much wider cross reactions described by Adant (1930) do not agree with general experience In this type of determinant group the terminal part seems less important than in the polypeptides and disaccharides, specificity is determined by the whole of the determinant group

Table X and Table XI illustrate the relative nature of specificity Some sera are far more strictly specific than others As a general rule the sera that form the heaviest precipitate with the homologous antigen are least specific

TABLE X

Cross reactions between synthetic antigens made from  , (*Jacobs*, 1937) *Upper line readings after 15 minutes at room temperature, lower line after night in cold*

Antisera to antigens made with horse serum protein, in which R *is* ↓		*Antigens made with chicken serum protein, in which* R *is*			
		O	CH_2	CO	−N=N−
O	Serum I	++ ++	=± ±±	±± ++	± ±
	Serum II	+± ++±	+ ++	Tr +±	± +
CH_2	Serum I	O ±	+ ++	O Tr	O Tr
	Serum II	+± +±	++ ++±	+± +±	F Tr Tr

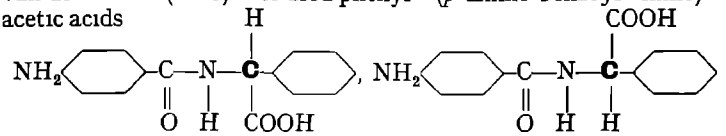
TABLE XI

Cross reactions of antisera to antigens, in which determinant groups are joined to horse serum protein by ureido and azo links, with antigens made with pig serum proteins, Mutsaars and Grégoire (1936)

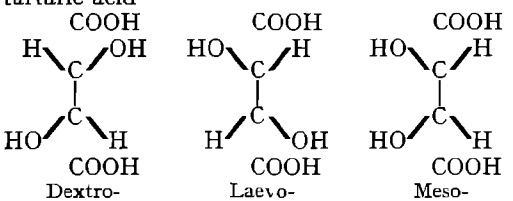
Anti serum		*Phenyl-ureido antigen*	*α-naphthyl-ureido antigen*	*Aniline azo antigen*	*α-naphthyl-amine azo antigen*
Phenyl-ureido serum	1	+++	++	+	O
,,	2	++	O	O	O
,,	3	O	O	O	O
,,	4	+++	++	++	O
α-naphthyl-ureido serum	1	O	+++	O	O
,,	2	O	+++	O	O
Aniline-azo serum	1	O	O	+	O
	2	++	++	+++	+
	3	+	O	++	O

2. *The Effect of Spatial Configuration*

Studies on the effects of spatial configuration on immunological character have produced very interesting results Landsteiner and van der Scheer (1928) first used phenyl—(*p*-amino-benzoyl-amino)—acetic acids



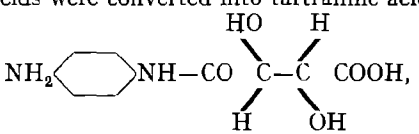
The dextro- and laevo-isomers differ only in the arrangement of the groups round the —C— atom in heavy print The two optically active forms and the inactive mixture of laevo- and dextro- were linked to proteins in the usual way and used as antigens There was some overlapping in the reactions of the resultant antisera with the two forms Thus the *d*-antiserum gave a slight precipitate with the *l*-antigen diluted 1/400, but a better precipitate with the *d*-antigen diluted 1/50,000 , inhibition tests with the simple haptenes also showed some overlapping As might be expected, the racemic antigen and haptene behaved like a mixture of the *d*- and *l*- compounds This immunological differentiation of optical isomers may be compared to their differential adsorption by wool (Chapter I) More specific results were obtained (Landsteiner and van der Scheer, 1929) with tartaric acid



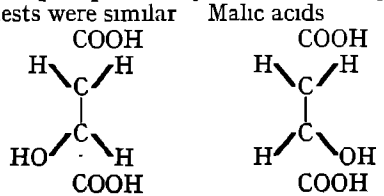
Dextro- Laevo- Meso-

The heavily printed bonds are in front of the plane of the paper , the dotted bonds behind

Here the inactive meso-acid is not a mixture of laevo- and dextro-acids These acids were converted into tartranilic acids, e g

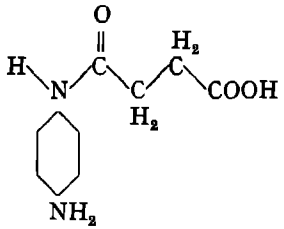


and these compounds coupled with protein in the usual way The *l*- and *d*-antisera both gave only very slight reactions with the opposite antigen, but stronger reactions with the *m*-antigen But the *m*-antiserum gave a precipitate only with the *m*-antigen The results with inhibition tests were similar Malic acids

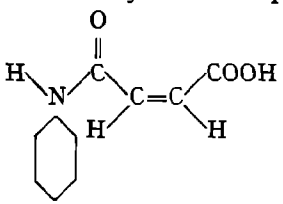


which contain only one asymmetric C atom had very weak inhibitory effect, each affecting the corresponding tartaric-acid reaction. Succinic acid, $COOH\,CH_2\,CH_2.COOH$, with no assymmetric C atom, had no inhibiting effect

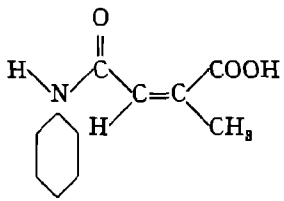
Antisera to *p*-amino-succinanilic acid



are inhibited by the cis-compounds

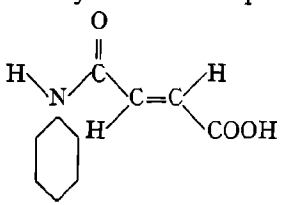


Maleic Acid

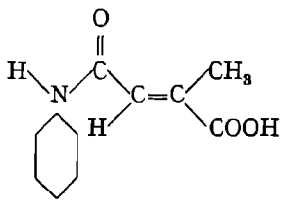


Citraconic Acid

but not by the trans-compounds



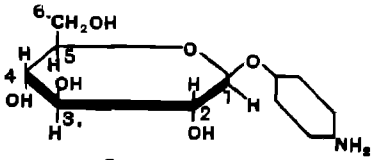
Fumaric acid



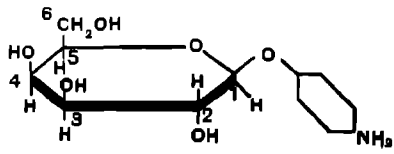
Mesaconic Acid

(Landsteiner and van der Scheer, 1934)

The effect of differences of spatial arrangement have been studied also with carbohydrate-containing antigens Avery and Goebel (1929) coupled proteins with *p*-amino-phenol-*β*-glucoside and *p*-amino-phenol-*β*-galactoside, and used these as antigens



β-Glucoside



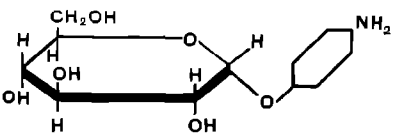
β-Galactoside

The hexose ring is at right angles to the plane of the paper The heavily printed bonds of the ring are in front of the paper and the light ones behind

They differ only in the arrangement of the —H and —OH at the 4th C atom of the hexose[1] Both precipitate formation with the resultant antisera and inhibition were highly specific, inhibition was produced by 0 2 c c of a 0 01 M solution of the homologous hexoside, but not by 0 2 c c of a 0 1 M solution of the heterologous hexoside

The effect of differences in the arrangement of —H and —OH at a single C atom is also illustrated by the work of Woolf, Marrack and Downie (1936) A glucuronic compound (euxanthic acid) attached to protein forms a precipitate with Type II anti-pneumococcal serum The formation of this precipitate is inhibited by salts of glucuronic acid, but not by salts of galacturonic acid (which differs from glucuronic acid in the arrangement at the No 4 C atom), nor by mannuronic acid (which differs in the arrangement at the No 2 C atom)

The α- and β-glucosides were also studied (Avery, Goebel, and Babers, 1932)



α–Glucoside

The distinction between these was not so sharp as between the glucoside and galactoside, the antisera gave precipitates with the heterologous antigens, though these were less heavy than with the homologous antigens An interesting point in connexion with this cross-reaction was that the precipitate formation by an antibody and the homologous antigen was inhibited only by the homologous simple glucoside, but the reaction with the heterologous antigen was inhibited by both homologous or heterologous glucoside (see Table XII).

TABLE XII

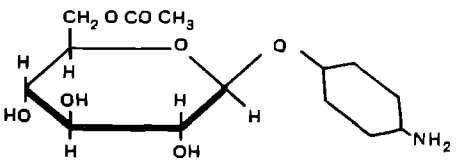
Inhibition of reactions of α- and β-glucoside antisera with homologous and heterologous antigens by homologous and heterologous simple haptenes (Avery, Goebel and Babers, 1932)

	Antiserum to α-glucoside		*Antiserum to β-glucoside*	
Test Antigen	*with α-glucoside haptene*	*with β-glucoside haptene*	*with α-glucoside haptene*	*with β-glucoside haptene*
α-glucoside	O	+	O	O
β-glucoside	O	O	+	O

[1] The ring structure of hexoses and method of representing them in perspective is discussed later, in the section on carbohydrates The representation of the hexoses as straight chains, on the flat, gives the arrangement round the No 4 C atom a false appearance of unimportance It is actually the terminal C atom The amino-phenol was attached by the glucoside link to the hexoses in order to provide an aromatic —NH_2 group by which they could be attached to proteins

Goebel, Avery and Babers (1934) have done further work on these lines using the disaccharides maltose, cellobiose and gentiobiose The reactions of the antisera obtained were determined by both the hexoses of the disaccharides, though the terminal hexose had the dominant effect, and by the stereo-chemistry of the bond between them. Thus the antiserum to the α-glucoside, maltose, gave more precipitate with the maltose antigen than with the β-glucoside, cellobiose, antigen (difference of link between hexoses) , it gave very little precipitate with the β-galactoside, lactose, antigen (difference both of terminal hexose and of link between hexoses) The cellobiose antiserum gave less precipitate with β-glucose antigen (only one hexose in common) than with cellobiose antigen The inhibition of these reactions of these antisera with homologous and heterologous antigens, by homologous and heterologous haptenes is illustrated by the example given in Table XIII

The reactions of such carbohydrate antigens may be modified by the introduction of substituents into the carbohydrate Goebel, Babers and Avery (1934) synthesized acetyl-β-glucoside antigens



The acetyl-β-glucoside antigen formed no precipitate with antisera to α-glucoside antigen and less with β-glucoside antiserum than was formed by the unsubstituted β-glucoside antigen Similarly, with the antiserum to the acetyl-β-glucoside antigen, the α-glucoside antigen formed no precipitate, and the β-glucoside antigen a reduced precipitate The inhibition cross-reactions were similar to those of the α- and β-glucosides

3 *Other Methods of introducing Determinant Groups*

Compared with the azo-method the possibilities of introducing new groups into proteins by other methods used up to now are very limited The chief interest of such compounds hitherto has lain in the effect of substitution on the original immunological character of the protein Halogens and nitro groups can be introduced into proteins probably mainly into the benzene ring of the tyrosine, ortho- to the —OH group (Bauer and Strauss, 1929) The resulting products have been reinvestigated by Wormall (1930) [1] The original immunological

[1] Nitro-proteins and proteins in which a diazo group —N=N—Cl is introduced by treatment with nitrite and hydrochloric acid can hardly be differentiated in precipitation tests (Landsteiner and Prasek, 1913, Wormall, 1930) Wormall suggests that in both the diazo and nitro-proteins the substituted aromatic rings have a quinoid structure and that the immunological relationship is due to this common change and not to the substituents

TABLE XIII

Reactions of antisera to mono- and di-saccharide antigens with homologous and heterologous antigens, final concentration 1/5,000, *and inhibition of reactions by homologous and heterologous simple haptenes, final concentration* 0 055*M* (*Goebel, Avery and Babers,* 1934)

Antisera to

Test antigens ↓	α-glucoside antigen	β-glucoside antigen	β-galactoside antigen	β-cellobioside antigen	β-maltoside antigen	β-gentiobioside antigen	β-lactoside antigen
α-glucoside	+++	+	O	±	++±	O	O
β-glucoside	++	++++	O	+++	±	+++	O
β-galactoside	O	O	+++	O	O	O	+±
β-cellobioside	±	++±	O	++++	±	++±	±
β-maltoside	+++	+++	O	+++	++++	++±	±
β-gentiobioside	±	++±	O	+++	±	++++	±
β-lactoside	O	O	++	++±	±	±	+++±

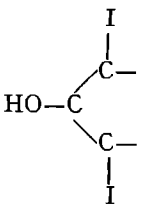
Inhibition of precipitate formation by antiserum to β-maltoside antigen, with homologous and heterologous antigens, by homologous and heterologous simple haptenes, final concentration 0 055 M

Hapten ↓	α-glucoside antigen	β-glucoside antigen	β-maltoside antigen	β-cellobioside antigen	β-gentiobioside antigen
p-amino-phenol α-glucoside	O	O	+++±	O	O
,, ,, β-glucoside	++±	O	+++±	O	O
,, ,, β-galactoside	++±	+++	+++±	++	+±
,, ,, β-cellobioside	++±	±	+++±	O	O
,, ,, β-maltoside	O	O	O	O	O
,, ,, β-lactoside	++±	+++	+++±	++	+±
,, ,, β-gentiobioside	++±	O	+++±	O	O
Control	++±	+++	++++	++±	++±

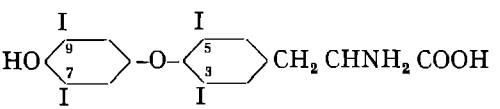
character was more or less completely destroyed and a new specificity common to proteins containing the respective " determinant groups " was found Iodo- and bromo-proteins, however, gave strong cross-reactions The precipitate formation by both iodo-proteins and bromo-proteins with corresponding antisera was inhibited by diiodotyrosine,

less efficiently by dibromotyrosine, and still less efficiently by dichlorotyrosine Potassium iodide, *o*-iodophenol, tyrosine and other amino-acids were without effect

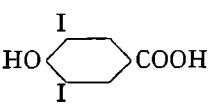
This work was extended by Snapper and Grunbaum (1936) They found that all aromatic compounds with a group



inhibited Thyroxine



inhibits, but thyronine, in which the 7, 9 iodine atoms are absent does not , nor does the aliphatic CH_2I CHOH CH_2I If the hydrogen of the —OH group of

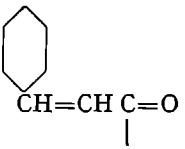


3 5 -diiodo-4-oxybenzoic acid is replaced by an acetyl group CH_3 CO— the compound still inhibits, though less well , but the ether, in which this hydrogen is replaced by $—CH_3$, does not

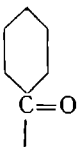
Various other chemical modifications have the effect of destroying the original immunological character of the protein and inducing a new " determinant group " specificity Acyl groups may be introduced into the —OH and $—NH_2$ groups of the protein (Landsteiner and Jablons, 1914) Some cross-reaction occurred between proteins containing different acyl groups Acetyl-protein ($CH_3CO—$) gives some complement fixation with antipropionyl (CH_3 CH_2,CO—) serum

but not with antibutyryl ($CH_3CH_2CH_2CO$—) and antivaleryl ($CH_3CH_2CH_2CH_2CO$—) sera, the aromatic anisoyl-

and cinnamyl-



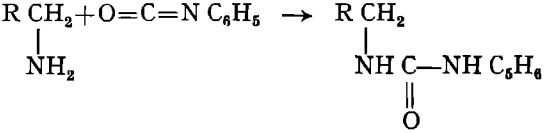
proteins do not give cross-reactions with those containing aliphatic acyl radicles (Landsteiner and Lampl, 1917, b) Medveczky and Uhrovitz (1931) have found that antibenzoyl-



protein serum will give precipitates also with *m*- and *p*-nitro-benzoyl-proteins in dilutions about as high as with benzoyl-proteins, and with α-brompropionyl, α-brom*iso*valeryl, and α-brom*iso*capronyl proteins With the last three antigens the highest dilution in which precipitation occurred increased with the molecular weight of the acyl group, this recalls the progressive efficiency of fatty acids of rising molecular weight, in inhibiting the reaction between amino-benzoic acid azo-protein and its antiserum (p 75)

Methyl groups may be introduced into the —OH, $-NH_2$ and = NH groups of proteins (Landsteiner, 1917) In complement fixation tests those methyl-proteins give cross-reactions with ethyl-proteins, but not with untreated protein, nor with nitro-, acetyl-or heat-coagulated proteins

Recently Hopkins and Wormall (1933) have introduced a new method of introducing "determinant groups", carrying a new immunological character, into proteins, which involves less violent treatment—the reaction of free $-NH_2$ groups of proteins with phenyl-*iso*-cyanate, forming a substituted urea



The reaction has the advantage that it can be carried out at pH 8 Although the substitution of amino groups was not complete the cross-precipitin reaction with untreated protein was much reduced Precipitate formation by the compound with its antiserum was inhibited by similar compounds of phenyl-*iso*-cyanate with lysine or ε-amino-*n*-hexoic acid, the phenyl-*iso*-cyanate compounds with other amino-acids were less effective (Hopkins and Wormall, 1934)

Antisera to antigens formed by the action of phenyl-*iso*-cyanate on proteins

$$\text{Protein—C(=O)—NH } C_6H_5$$

reacted weakly with test antigens formed by coupling diazotized aniline to proteins (Protein $-N=N-C_6H_5$) The two types of antigen differ in the site and mode of attachment of the foreign benzene ring (Table XI, Mutsaars and Grégoire, 1936)

Gaunt, Higgins and Wormall (1935) introduced another method of attaching new groups to proteins, without drastic treatment at a hydrogen ion concentration near neutrality, through the action of benzyl-carbonyl chloride The antisera obtained by immunizing with the product are specific for the altered protein

Formaldehyde acts on free $-NH_2$ groups

$$-NH_2 + 2H\,CHO \;=\; -NH_2\,(H\,CHO)_2$$

Von Eisler and Lowenstein (1912) found that protein, so treated, behaved as untreated protein both in precipitin and anaphylactic reactions Landsteiner and Lampl (1917, a) obtained similar results, treated proteins retained their species specificity However, Horsfall (1934) found that antisera to formaldehyde-treated proteins would form precipitates with formaldehyde-treated proteins of other species, but with these heterologous antigens the titre was much lower Precipitate formation was not inhibited by formaldehyde-treated amino-acids Anaphylactic shock was produced in guinea-pigs, passively sensitized by formaldehyde-treated proteins, both by injection of homologous protein, untreated with formaldehyde, and of heterologous protein treated with formaldehyde Horsfall used smaller quantities of formaldehyde than did Landsteiner and Lampl, but allowed the reaction to go on for a longer time The contrast between treatment with formaldehyde and other treatments such as that with phenyl-*iso*-cyanate remains, although this work of Horsfall makes it less striking

Blumenthal (1936) found that when serum albumin was reduced by thioglycollic acid the product did not precipitate all the antibody from an antiserum to untreated serum albumin, re-oxidation with hydrogen peroxide did not affect the amount of antibody precipitated by this reduced albumin On the other hand, reduction of egg albumin with thioglycollic acid did not affect its serological behaviour This is correlated with the fact that serum albumin contains disulphide (—S—S) groups which can be reduced to sulphydryl (—S—H) groups by thioglycollic acid, whereas undenatured egg albumin contains no such reducible groups The essential change produced by this reduction of serum albumin might be a loosening of the bonds between contiguous peptide chains or laminae which are held together by —S—S— groups

In the haemoglobins we have examples of natural proteins which have a common non-protein group attached to different protein

fractions The immunological character of the protein (globin) is altered thereby, for haemoglobin and globin, although they give cross-reactions are not immunologically identical (Hektoen and Schulhof, 1927) [1] But the haemoglobins do not show a common immunological character due to the prosthetic group Also the reaction between a haemoglobin and its antiserum is not inhibited by haematin (Heidelberger and Landsteiner, 1923), which might be expected to act as a simple haptene , and antisera to haemoglobin give precipitates with methaemoglobin, carboxyhaemoglobin, and sulphaemoglobin in the same titre, in spite of the change in the prosthetic group It appears that the prosthetic group does not give globin a " determinant group " specificity This may be due to the small number of such groups present One haemoglobin molecule contains four prosthetic groups, while some twenty active groups per molecule may be introduced by the azo-method

B Natural Protein Antigens

Any theories which may be advanced to account for the interaction of antigens and antibodies must be compatible with the known facts concerning the immunological characteristics of naturally occurring proteins There is no evidence that such proteins contain characteristic " determinant groups " such as those introduced in the artificial antigens we have been considering They appear to differ in the proportions and arrangements of the amino-acids of which they are built and the consequent structural differences

In some cases proteins derived from different species which have similar physical properties and chemical constitution may appear identical immunologically The globulins obtained from cantaloupe seeds and squash seeds have similar crystal forms and optical properties , the elementary analyses, content of amino-acids and partitions of nitrogen by the van Slyke method, agree very closely They are indistinguishable by the anaphylaxis reaction (Jones and Gersdorff, 1923) But more delicate methods may reveal immunological differences between proteins which were supposed to be identical Thus Dakin and Dale (1919), using the isolated guinea-pig uterus method, were able to demonstrate a difference between the albumins of hens' and ducks' eggs, although previously they had been found identical by both precipitin and anaphylactic reactions Also Hooker and Boyd (1936) showed that hen egg albumin would precipitate 0 39 mg of antibody N from 1 c c of anti-hen-egg-albumin serum whereas duck egg albumin would only precipitate 0 067 mg

With other proteins immunological differences can be readily detected between samples from different species Fibrinogens derived from various mammals give cross-reactions , but the antiserum to a fibrinogen from any species gives a precipitate with fibrinogen from this species in higher dilution than with the fibrinogen of another

[1] The discrepant results of Ottensooser and Strauss (1928) may be ascribed to the use of denatured globin

species (Hektoen and Welker, 1927) Thyreoglobulin (Hektoen, Fox, and Schulhof, 1927) and lens protein (Hektoen and Schulhof, 1924) behave in the same way Species differences between serum proteins are more striking Cross-reactions are obtained in the precipitin reaction between preparations obtained from closely related species, even between far separated species such as horse and man, if strong antisera are used undiluted But quantitative differences are always demonstrable, and cross-reactions, even between the serum proteins of fairly closely related species such as ox and horse, do not appear to occur in anaphylactic reactions However, these proteins are distinguishable by such analytical methods as the partition of nitrogen by the procedure of van Slyke (Hartley, 1914, c, Crowther and Raistrick, 1916) [1]

Haemoglobin is another example of the relative nature of species specificity Heidelberger and Landsteiner (1923) found cross-precipitin reactions, other than a minimal trace, only between the haemoglobins of closely related species, e g horse and donkey But they found evidence of a relationship between haemoglobins of different origin by means of the inhibition reaction, the formation of a precipitate by horse haemoglobin and its antiserum was inhibited almost completely by excess of dog haemoglobin Hektoen and Boor (1931) have shown that some antihaemoglobin sera will give precipitates with haemoglobin from a wide range of species, when used undiluted, although when diluted one-third they give precipitates only with the homologous antigen

Investigations by the method introduced by Dakin and Dudley (1913) suggest that the immunological differences between proteins of apparently similar constitution are due to different arrangements of the constituent amino-acids in the molecules The principle of the method is to keep the proteins in N/2 alkali at 37° C for about three weeks By this time the optical activity of the protein is found to have been greatly reduced, and on hydrolysis certain of the amino-acids are found to be no longer optically active (have been racemized) The theory is that only those amino-acids which are situated at the

[1] It was suggested by Rimington (1929) that the carbohydrate fraction of serum proteins might play some part in determining their immunological character This carbohydrate, however (glucosamine-dimannose, Rimington, 1931), appears to be present both in albumin and globulin, and in the proteins of the sera of different species It does not give a precipitate with antisera to horse-serum globulin, nor inhibit precipitate formation by such sera and horse-serum globulin A carbohydrate obtained from egg albumin by Rimington's method did not react with a strong anti-serum to egg albumin (Ferry and Levy, 1934) Sevag and Seastone (1934) obtained a carbohydrate from egg white This carbohydrate did not form a precipitate with an antiserum to egg white, which formed a precipitate with egg albumin at a dilution of 1/200,000 Nor did it inhibit the formation of a precipitate by this serum and egg white or the protein fractions of egg white However, injection of 0 5 mg of this carbohydrate caused anaphylactic shock in guinea-pigs sensitized with egg white It appears that this carbohydrate may act as an antigen, but is not concerned in the immunological behaviour of the egg proteins.

ends of the peptide chains resist this change, this has been demonstrated by Levene, Steiger, and Marker (1931) and Levene and Steiger (1932) working with polypeptides If therefore in two proteins, so treated, different amino-acids are found to have resisted racemization, it is inferred that the arrangement of amino-acids in the chains of the two differ Actually definite differences were found in this way between the crystalline egg albumin of hens' and ducks' eggs (Dakin and Dale, 1919), which can be distinguished by the anaphylactic reaction, and by quantitative precipitin reactions Similar differences were also found by Dudley and Woodman (1915) between caseinogen of cow and sheep milk These two again cannot be distinguished by precipitin reactions or anaphylaxis as usually performed, but the more delicate form of anaphylactic reaction has not been tried

It is probable that the theory of this method is not so simple as given above Long exposure to alkali splits up the protein with evolution of ammonia It is also hardly conceivable that 85 per cent of the leucine, nearly all the proline, and the greater part of the alanine, arginine and histidine all occupy terminal positions in peptide chains in duck egg albumin, as would be the case according to the theory and the results of Dakin and Dale Nevertheless, if the results are constant, differences found by this method must be evidence of differences of structure in proteins in which no difference could be detected by other methods It is probable that such differences also exist between the serum proteins of different species

Taking into consideration the importance of the terminal amino-acid in the experiments of Landsteiner and van der Scheer (1932) already mentioned, in which peptides were introduced as " determinant " groups, it appears probable that the immunological character of natural proteins is determined by the arrangement of amino-acids on the surface of the molecule It may be either that individual acids act as " determinant groups ", in which case the character would depend on their distribution on the surface, or that several may together form an " active patch " with a characteristic spatial distribution of inter-molecular forces Such a patch might be more or less completely altered by the molecular rearrangements taking place in denaturation

Since most of the modifications of protein which have been found to alter its immunological character (e g addition of halogens, nitration, formation of azo compounds) have affected the benzene rings, tyrosine has been regarded as playing an especially important part in determining the immunological character of proteins It is very possible that tyrosine with its stable ring and polar —OH group is of more importance than the aliphatic amino-acids But the modifications in question must alter the structure of the protein considerably, apart from the effects on the benzene rings, and the observations of Hopkins and Wormall (1933) show that the immunological character of a protein may be altered by a process that does not affect the benzene rings

In connexion with the " specificity " of proteins it may perhaps be claimed that over-much stress has been laid on the "species" aspect of immunological characters It suggests that chemically different proteins of the same species may have some immunological characters in common There is no evidence of this Thus fibrinogen (Hektoen and Welker, 1927), serum globulin, and serum albumin, which differ chemically, do not give cross-reactions by either the precipitin test (Hektoen and Welker, 1924) or by anaphylaxis (Dale and Hartley, 1916) Egg white has been shown to contain four immunologically distinct proteins besides conalbumin, which appears to be identical with serum albumin (Hektoen and Cole, 1927, 1928), and milk three besides lactoglobulin, which appears to be immunologically identical with serum globulin (Wells and Osborne, 1921) Chemical differences (Table XIV) between the milk proteins, caseinogen, lactalbumin, and

TABLE XIV

Diamino and cystine N of various proteins, estimated by the van Slyke method, as per cent of total N

	Arginine	*Histidine*	*Lysine*	*Cystine*
Caseinogen[1]	7 51	4 24	7 86	1 95
Lactalbumin[1]	7 27	4 20	13 08	2 34
Lactoglobulin[1]	10 94	4 24	9 16	1 85
Ox-serum albumin[2]	10 4	6 7	16 3	3 5
Horse ,,	10 0	6 1	16 0	3 1
Ox-serum pseudoglobulin[1]	10 56	3 70	8 16	1 82
,, ,, [2]	10 8	4 8	9 6	1 9
Horse ,, [2]	8 9	5 8	9 8	1 7
Ox-serum euglobulin[1]	10 81	3 78	8 13	2 02
,, ,, [2]	11 6	3 8	9 2	2 0
Horse ,, [2]	8 5	5 0	9 9	1 7
Fibrin[3]	13 9	4 8 3 4	11 5	2 6
Haemoglobin[4]	8 1	8 8	12 0	0 0
Thyreoglobulin[5]	16 4	11 6	4 3	0 97
Cantaloupe seed globulin[6]	28 4	6 2	3 4	0 78
Squash ,, ,, [6]	27 5	6 7	3 85	0 79

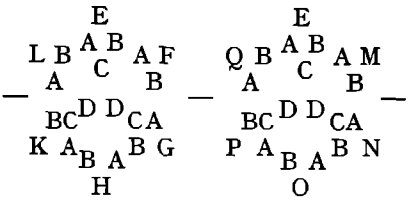
[1] Crowther and Raistrick (1916)
[2] Hartley (1914, c)
[3] Mitchell and Hamilton (1929)
[4] Plimmer and Rosedale (1925)
[5] Eckstein (1926)
[6] Jones and Gersdorff (1923)

lactoglobulin are shown by the van Slyke method (Crowther and Raistrick, 1916) Thyreoglobulin, lens proteins, and haemoglobin are immunologically different from serum proteins

Block and his colleagues (1934), have suggested that a common feature of similar proteins in different species is the ratio of the constituent basic amino-acids to one another Thus iron, arginine, histidine and lysine, expressed in terms of molecules occur in the ratios 1 3 8 9 in horse, sheep and dog haemoglobin, whereas the ratio iron cystine varies widely in the haemoglobins This common feature may account for cross reactions, while the diversity in other respects accounts for species differences

It was pointed out in the first chapter that if the amino-acids in proteins are arranged in a regular fashion there must be nodes or

patches in which certain amino-acids recur together. These two schemes may be represented diagramatically as ABCDE ABF .. ABCG ABDH ABCK ABL ABCDEM ABN and



It may be supposed that such localised recurring groups of amino-acids form the determinant groups of natural proteins comparable to the amino-acids in the polypeptide antigens of Landsteiner and van der Scheer (1934, b) If the protein molecules have the laminar structure suggested by Wrinch (1937) proteins would also be differentiated by the special arrangement of such groups relative to one another and by the distribution of —OH and side chains of amino-acids on the sides of the molecule

Supposing that such groups of amino acids form the determinant groups of natural proteins it might be hoped that a precipitation reaction, in which the antigen was a protein, might be inhibited by by-products of digestion of proteins by enzymes (cf the inhibition of the reaction of S S S III with the homologous serum by breakdown products of S S S III, Heidelberger and Kendall, 1933) Landsteiner and colleagues (1930–1931, 1932–1933) did not obtain such inhibition with the usual anti-protein sera But when heteroalbumoses, obtained by peptic digestion, were used for immunization, antisera were obtained which gave precipitates with these heteroalbumose fractions and with the unchanged protein The formation of these precipitates was inhibited by albumose fractions which themselves gave no or very slight precipitates with the sera, and would pass through a collodion membrane

We may here revert to the question of the presence of separate fractions eu- and pseudoglobulin in untreated serum Harris and Eagle (1935) immunised rabbits with untreated human and horse serum There was evidence that in the sera of these rabbits there were separate antibodies to euglobulin (precipitable by 33 per cent saturation with ammonium sulphate and water insoluble) and pseudoglobulin (not precipitable by 33 per cent saturation and water-soluble) When the rabbit sera were exhausted of antibody to horse or human euglobulin by repeated addition of euglobulin, they would still give precipitates with horse or human pseudo-globulin, and vice versa These experiments suggest that there are present in the globulin of untreated serum separate fractions corresponding to eu- and pseudo-globulin, which act as antigens and give rise to distinct antibodies, even though they may not differ in particle size.

Denatured Proteins

The immunological character of proteins may be altered by the processes classed as denaturation Wu, Ten Broeck and Li (1927) have shown that, when egg albumin was denatured by heat in neutral, acid or alkaline solution, by acid, alkali or alcohol, the products were partially equivalent They still might give some degree of cross-reaction with antisera to the native protein The change by heat is a gradual one, the ability to give a precipitate with antiserum to the natural protein gradually disappears as the time or temperature of heating is increased (Furth, 1925, 1926) Spiegel-Adolf (1926) has confirmed the observation of Obermayer and Pick that a heat-denatured protein would inhibit the formation of a precipitate by natural protein and its antiserum, though the denatured protein would no longer give a precipitate with this serum

Denatured proteins do not gain a new group character, e g heat-denatured horse-serum proteins will not give cross-reactions with heat-denatured chicken-serum proteins

The change in immunological character is not due to loss of an essential constituent, for Spiegel-Adolf (1926) found that the heat denaturation of serum albumin can be reversed, and that the recovered albumin is immunologically identical with the natural albumin Similar reversal of denaturation was also claimed by Anson and Mirsky (1931), and Miller (1933) found that the reversed protein did not differ in serum reactions from native protein These experiments have been criticized by Hewitt (1934), who considers that all the protein is not denatured by the procedure used by Anson and Mirsky, and that the " reversed protein " is merely undenatured protein which has been separated from the denatured fraction

The rearrangement of the protein molecule from a structure such as Wrinch's cyclol to a fibrous structure, as indicated by Astbury's work (see Chap I), would involve a complete change in the spatial distribution of the groups of amino-acids, which it is suggested act as determinant groups, sufficient to account for a fundamental change of immunological character As suggested in Chapter I there may be different degrees of denaturation, depending on the degree to which peptide chains have become straight and parallel, both the degree of immunological change and of irreversibility may depend on the extent of this process

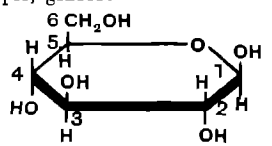
C Polysaccharides

One of the striking developments of recent work in immunology is the discovery of numerous polysaccharides which react specifically with appropriate antisera Many of these give precipitin reactions in such extreme dilutions that it is most unlikely that the reactions are due to a contamination A few have been so highly purified and subjected to such varied manipulations, without effecting their immunological character, that such a supposition is out of the question; this is particularly the case with the specific soluble substance of Type III pneumococcus (S S S III)

In the case of five polysaccharides, S S S III, S S S VIII,* the purified polysaccharide of gum acacia, the polysaccharide of Friedlanders' bacillus (Type A) and the gum of *Penicillium luteum*—the structure has been worked out sufficiently for it to be possible to relate this to their immunological behaviour The first of these, S S S III, is built up of aldobionic acid molecules united by a glucoside link[1] These aldobionic acid molecules consist of one molecule of glucuronic acid and one of glucose, united by a glucoside link involving the reducing group of the glucuronic acid (Heidelberger and Goebel, 1927) This is attached to the No 3 or 6 carbon atom of the glucose These aldobionic-acid molecules are joined together by a glucoside link involving the reducing group of the glucose , the —COOH group of the glucuronic acid is left free

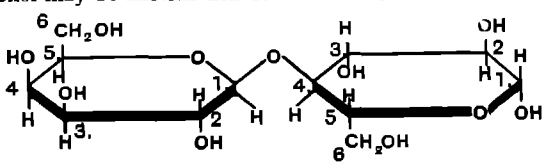
The aldobionic acid of S S S VIII is identical with that of S S S III (Goebel, 1935) But the polysaccharide S S S VIII is not made up wholly of aldobionic units, as it contains glucose which is set free before the aldobionic acid is hydrolysed

[1] It has been shown that in the stable form of the aldo-hexoses (glucose, mannose, and galactose, for example) the first five carbon atoms together with an oxygen atom form a hexagonal ring The molecule is conveniently represented as, for example, glucose



The ring is supposed to be at right angles to the plane of the paper , the thin bonds of the ring being behind the plane of the paper and the thick bonds in front

These hexoses can combine with an alcohol by elimination of one molecule of water from the —OH group attached to the No 1 C atom of the hexose and the —OH group of the alcohol, forming a hexoside (glucoside, galactoside, &c , as the case may be) The reducing power of the hexose depends on the —OH group of the No 1 C atom , this group is blocked in a hexoside and will not reduce Fehling's solution unless the hexoside is hydrolysed The second alcohol may be another hexose as in lactose

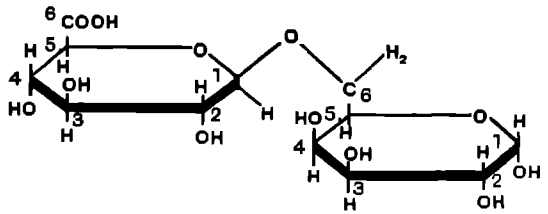


Galactose **Glucose**

With the orientation of the galactose molecule shown in the figure, when the O atom of the hexoside link is below the plane of the ring the compound is called an α-hexoside , when this O atom is above this plane the compound is called a β-hexoside A series of hexose molecules may be linked in this way forming a long chain Cellulose consists of chains of 100–200 glucose molecules united in this way Heidelberger and Kendall (1932) suggest that S S S III contains twelve aldobionic acid units similarly united

Acids formed by oxidation at the No 6 C atom of hexoses to —COOH are called hexuronic acids (e g glucuronic, galacturonic)

From gum acacia a polysaccharide can be prepared which gives a precipitate, in extreme dilution, with antisera to Type II and Type III pneumococci (Heidelberger, Avery, and Goebel, 1929) On hydrolysis of this polysaccharide an aldobionic acid is obtained composed of glucuronic acid and galactose (Heidelberger and Kendall, 1929, b), the structure, according to Challinor, Haworth, and Hirst (1931) and Hotchkiss and Goebel (1936), is that shown below (galactose–6–β–glucuronide) This aldobionic acid is hydrolysed



Glucuronic acid Galactose

more easily than that of S S S III , this suggested that the glucoside link was not attached to the same atom of the hexose in the two acids. Hotchkiss and Goebel (1936) have synthesized the heptacetyl methyl ester of the acid in which glucuronic acid is attached to the No 6 carbon atom of glucose (gentiobiuronic acid) , this differs from the heptacetyl methyl ester of the aldobionic acid prepared from S S S III in melting point and optical rotation The cross-reaction of the polysaccharide of gum acacia with S S S III serum, is presumably due to glucuronic acid similarly attached in the two polysaccharides

The polysaccharide of Friedlander's bacillus (Type A), besides other constituents, yields an aldobionic acid on hydrolysis , the —COOH of this acid is free in the polysaccharide This aldobionic acid, although composed of glucose and glucuronic acid differs from that of S S S III, its rotary power is higher and it is more easily hydrolysed (Goebel, 1927) It must be assumed that the reducing group of the glucuronic acid is attached to a different C atom of the glucose This polysaccharide is immunologically distinct from that of Types B (Julianelle, 1926 = Type E of Avery, Heidelberger, and Goebel, 1925) and C of Friedlander's bacillus , also from S S S II, which, however, gives cross-reactions with the polysaccharides of Type B

The fourth polysaccharide of which the structure is known is luteic acid, which Raistrick and Rintoul (1931) obtained from *Penicillium luteum* It is built up of units, each of which is composed of two molecules of glucose and one molecule of malonic acid The glucose molecules are joined by a glucoside link, the malonic acid forming an ester with one of them The glucose molecules of the units apparently form a chain, connected by glucoside links, the remaining —COOH group of the malonic acid being free. The attachment of the acid in this polysaccharide differs from that of the glucuronic acid in the

three others considered, and resembles that of phosphoric acid to pentose in nucleic acids, and of sulphuric acid in chondroitin and mucoitin sulphuric acids

Many, but not all, of the polysaccharides studied contain uronic acids, the —COOH groups of which appear to be free These uronic acids unquestionably play an important part in determining the immunological behaviour comparable to the predominant effect of acids already noted This is obvious in the case of S S S III and S S S VIII, in which the presence of the same aldobionic acid in both gums gives rise to a strong cross-reaction (Goebel, 1935) Also in the case of the gums (acacia, cherry, mesquite, see Table XV), which give precipitates with Type II antipneumococcal serum Marrack (1936), formation of these precipitates is inhibited by glucuronic acid salts, not however by salts of galacturonic and mannuronic acids Also, again, in the formation of precipitates by glucuronic acid compounds coupled with protein when these are mixed with Type II (Woolf, Marrack and Downie, 1936, and Goebel, 1936, a and b), and Type III and VIII serum (Goebel, 1936, b) Chow and Goebel (1935) found that esterification of the uronic acid of S S S I abolished its reaction with Type I antipneumococcal sera

The cross-reactions that occur among these polysaccharides is well accounted for by the presence of common features The work already quoted on the effect of structural difference on the specificity of synthetic antigens which contain sugars gives an indication of the types of difference in polysaccharides which may be expected to give rise to differences in immunological behaviour S S S III and S S S VIII differ in the spacing of the determinant aldobionic acid groups Gum acacia differs from S S S III, not only in this spacing and in the presence of galactose in place of glucose, but also in the point of attachment of the glucuronic acid to the hexose—a difference comparable to that between maltose and gentiobiose

A possible further source of such differences may be sought in substitutions of atoms in the sugar units of the polysaccharides For example, an acetyl can be introduced into the glucose of the β-glucoside synthetic antigens, as already described With antisera to the non-acetylated compound the non-acetylated forms more precipitate than does the acetylated compound and vice-versa (Goebel, Babers and Avery, 1934) This is in accord with the behaviour of natural S S S I containing acetyl groups, and of the same polysaccharide from which acetyl groups have been removed by treatment with alkali as in the earlier methods of preparation (Avery and Goebel, 1933) Heidelberger and Kendall (1935, b) found that, when methyl groups were introduced into S S S III, the product precipitated only part of the precipitin for S S S. III in Type III anti-pneumococcal sera

It is surprising that other substitutions should not have more effect on the specificity of the products Chow and Goebel (1935) found that the derivatives of S S S I in which a methyl group was substituted on the primary amino as well as a methoxy group covering a

hydroxyl group, still reacted readily with antiserum to Type I pneumococcus In the process of making a synthetic antigen containing S S S III and protein, Goebel and Avery (1931) prepared the mono-nitro-benzyl ether of S S S III, the corresponding amino compound, and coupled this amino compound with horse globulin These compounds reacted with Type III antipneumococcal serum as though the reactivity of the S S S III contained was unaffected It should be noted that such cross-reactions between substituted S S S III and S S S III are not comparable to cross-reactions between synthetic glucoside antigens, in all of which the substitution is at the No 1 C atom These reactions of glucoside antigens with the corresponding sera are not inhibited by unsubstituted glucose

D Lipins

The part played by lipins in immunity reactions is of considerable practical importance , but the study of the relation of the immunity reactions of the substances grouped under this heading to chemical constitution is much more difficult than in the case of proteins and polysaccharides Lipins usually occur in mixtures and are exceedingly difficult to separate and obtain pure Some indication of this is given by the fact that in the purified lecithins used by Levene, Landsteiner and van der Scheer (1927) the ratio of nitrogen to phosphorus varied by as much as 20 per cent from the theoretical value Most commonly the highly unsatisfactory complement fixation reaction is employed, and when flocculation reactions are obtained it is highly uncertain, owing to the fact that the lipins do not form true solutions, whether lipin that is found in the precipitate is the actual antigen or merely carried down with a precipitate formed by some other substances , as, for example, cholesterol is carried down with the precipitates formed by protein antigens and their antibodies, or by diphtheria toxin and antitoxin

It is obvious that among the very varied substances grouped as lipins there are adequate chemical differences to account for differences of immunological behaviour Even among substances of the same class such as the lecithins there is considerable variation in the fatty acids of the molecules It is unlikely that immunological methods would distinguish between the saturated fatty acids of higher molecular weight, since the lower acids, when attached to proteins, give cross-reactions But it is more possible that acids of various degrees of unsaturation (such as oleic $C_{18}H_{34}O_2$, linoleic $C_{18}H_{32}O_2$, linolenic $C_{18}H_{30}O_2$, and arachidonic $C_{20}H_{32}O_2$) present in lecithins should differ immunologically between themselves and from the saturated acids Also the phosphoric acid of lecithin may be attached to a terminal carbon atom of glycerol (α form) as in brain lecithin, or to the middle carbon atom (β form) as in egg lecithin (Rae, 1934) Apart from these differences between the individual lipins, the interaction between cholesterol and lecithin or fatty acids (Leathes, 1925 ; Adam and Jessop, 1928) suggests the possibility that lipin complexes may be formed with immunological characters

TABLE XV —*Composition of certain polysaccharides.*

	[α]*d*	*Acid equivalent*	*C per cent*	*H per cent*	*N per cent*	*Reducing power on hydrolysis as glucose per cent*	*Constituents identified*	*Titre with homologous serum*	*Cross Reactions*
Pneumococcus, Type I (Avery and Goebel, 1933)	+265° to 277°	576	42 55	6 58	4 85 Amino N 2 22	32	Galacturonic acid (uronic anhydride 56 per cent) Amino sugar Acetyl, one group per equivalent, not attached to NH_2 Basic unit a trisaccharide containing two uronic acid molecules and an amino sugar	1/6,000,000	
(Heidelberger, Kendall and Scherp 1936)		650			4 62 Amino N 2 0				
Pneumococcus, Type I deacetylated (Heidelberger, 1927, Avery and Goebel, 1933, Heidelberger, Kendall and Scherp, 1936)	+297°	535	40 33	6 23	5 05 Amino N 2 5	27 6	Uronic anhydride 65 per cent	1/6,000,000	Does not precipitate from antisera to Type I Pneumococcus all the antibody precipitable by the acetylated polysaccharide
Pneumococcus, Type II (Heidelberger, 1927, Heidelberger, Kendall and Scherp, 1936)	+54° to +58°	970 (600)	(45 8)	(6 4)	0 14 to 0 73	86 to 95	Glucose Aldobionic acid Uronic anhydride 17 6 to 19 8 per cent	1/5 000,000	No reaction with antisera to Types I and III Precipitate with antisera to Friedländer Type B to 1/2,000,000

TABLE XV —*Composition of certain polysaccharides*—contd

	[α]d	*Acid equivalent*	*C per cent*	*H per cent*	*N per cent*	*Reducing power on hydrolysis as glucose per cent*	*Constituents identified*	*Titre with homologous serum*	*Cross Reactions*
Pneumococcus, Type III (Heidelberger and Goebel, 1927, Heidelberger, Kendall and Scherp, 1936)	−36 0	330 to 350	(42 6)	(5 6)	0 08	84	Aldobionic acid (glucose and glucuronic acid) Uronic anhydride 51 per cent	1/6,000,000	Precipitate with antisera to Pneumococcus Type VIII under 1/50,000
Pneumococcus, Group IV, Type IV (Heidelberger and Kendal, 1931)	+30	1,550			5 5 Acetylated amino N	71	Amino sugar Glucose Acetyl	1/2,000,000	
Pneumococcus, Type VIII (Goebel, 1935)	+125°	750			0	76	Glucose, 7 Mols Aldobionic acid (glucose and glucuronic acid), 2 Mols	1/8,000,000	Precipitate with antisera to Pneumococcus Type III to 1/4,000,000
Pneumococcus, species specific (Tillet, Goebel and Avery, 1930)	+42°	1,050			6 1 Acetylated amino N 3 7 Free amino N 0 9		Amino sugar derivative Acetyl Phosphoric acid		
Friedländer, Type A (Goebel, 1927)	−100°	430	43 95	6 0	0 0	65	Glucose, 1 Mol Aldobionic acid, 1 Mol A dissaccharide, 1 Mol	1/2,000,000	

Friedländer, Type B (Goebel, 1927)	+100°	680	44 6	6 1	0 0	70	Glucose Aldobionic acid	1/2,000,000	Precipitate with antisera to Pneumococcus Type II to 1/2,000,000
Friedländer, Type C (Goebel, 1927)	+100°	680			0 0	70	Glucose Aldobionic acid		
Gum Acacia, weakly hydrolysed (Heidelberger and Kendall, 1929, b, Butler and Cretcher, 1929, Challinor, Haworth and Hirst, 1931)	−10 7°	800			0 8	68	Galactose Aldobionic acid (galactose and glucuronic acid) Another acid		Precipitate with antisera to Pneumococcus Types II and III to 1/1,000,000
Cherry Gum, weakly hydrolysed (Butler and Cretcher, 1931)							Arabinose Xylose Mannose, 3 Mols Galactose, 6 Mols Glucuronic acid, 2 Mols Nucleus of 2 Mols of Glucuronic acid 1 Mol of Mannose		Precipitate with antisera to Pneumococcus Type II to 1/1,000,000
Mesquite Gum, weakly hydrolysed (Anderson and Otis, 1930)							l-Arabinose d-Galactose, 3 Mols Methanol, 1 Mol Glucuronic acid, 1 Mol Nucleus of 1 Mol of Methoxy-glucuronic acid and 1 1 Mol of Mannose		Precipitate with antisera to Pneumococcus Type II to 1/100,000

TABLE XV—*Composition of certain polysaccharides*—contd

	[α]*d*	*Acid equivalent*	*C per cent*	*H per cent*	*N per cent*	*Reducing power on hydrolysis as glucose per cent*	*Constituents identified*	*Titre with homologous serum*	*Cross Reactions*
Flaxseed Mucilage, weakly hydrolysed (Anderson and Crowden, 1930)							l-Xylose l-Arabinose Glucose Galactose Nucleus of 1 Mol of galacturonic acid and 1 Mol of l-rhamnose		Slight precipitate with antisera to Pneumococcus Type II
Tubercle Bacillus, human, from medium (Heidelberger and Menzel, 1932)									
Fraction B	85°–95°	About 7,000			0 1	87	d-Arabinose Mannose	1/50,000	
Fraction C		About 2 000			0 6 to 0 8	61	d-Arabinose	1/50,000	
B C G, from bodies of bacteria (Chargaff and Schaefer, 1935–1936)	+77 4	1,244			0 8 P 0 9	77	d-Mannose 46 per cent d-Arabinose 8 per cent No galactose No uronic acid Inositol	1/1,000,000	
Tubercle bacillus, human, polysaccharide of purified wax (Roberts and Anderson, 1932)							d-Arabinose Mannose Galactose	1/3,000,000	

Tubercle bacillus, human polysaccharide of phosphatide (Anderson, 1932)							Inosite } equal Mannose } parts Other sugars		
Tubercle bacillus, bovine and avian, polysaccharide of phosphatide (Anderson, 1932)							Inosite Mannose		
B dysenteriae (Shiga) (Morgan, 1936)	+98°	9,000	41 9	6 35	1 61	97	Hexosamine Acetyl (attached to NH_2 group), 5 per cent No pentose, nor uronic acid	1/12,000,000	Inhibits haemolysis of sheep's red blood corpuscles by antiserum to *B dysenteriae* (Shiga)
"A" substance of horse saliva (Landsteiner, 1936, b)	+10°		44 6	6 91	7 08	57	Hexosamine Galactose Acetyl, 9 4 per cent No uronic acid		1/4,000 γ inhibits haemolysis of sheep's red corpuscles 1/1,000 γ inhibits isoagglutination
"A" substance of pepsin (Landsteiner and Chase, 1936)	+16		46 9	6 62	6 16	70 7	Glucosamine, 27 per cent Galactose Acetyl, 9 9 per cent No pentose, nor uronic acid		Approximately equal to "A" substance from horse saliva
Polysaccharide from urine, Group A (Freudenberg and Eichel, 1934)							Galactose, 33 per cent Acetyl, 10 per cent. Acetylated amino sugar		1/200 γ inhibits haemolysis of sheep's red corpuscles 5–10 γ inhibits isoagglutination

different from those of the constituent fractions, although the work of Hughes (1935) indicates that the interaction on surfaces is merely the result of the effect of cholesterol on the orientation of lecithin

The most important work that has been done with isolated lipins is that done with synthetic distearyl lecithin and with sterols, since these substances may be regarded as pure

Weil and Besser (1931) found that distearyl lecithin gave rise to antibodies when injected with pigs' serum[1] The synthetic lecithin is an α-compound (Grun and Limpacher, 1926) The sera obtained gave moderate complement fixation with distearyl lecithin, only very weak cross-reactions were obtained with Merck's lecithin Maier (1933), however, did not find such a clear distinction between these two antigens Levene, Landsteiner, and van der Scheer (1927) injected five rabbits with purified egg lecithin, the serum of only one of these reacted with the antigen and this one but feebly These sera, however, gave weak complement fixation reactions with Merck's lecithin, this was not accounted for by the cholesterol in the Merck's lecithin, for the purified lecithins did not fix complement even when cholesterol was added Injections of Merck's lecithin produced antisera which gave good flocculation and complement fixation reactions with Merck's lecithin (as had been found by Sachs and Klopstock, 1925) but not with purified lecithin Other attempts to obtain antisera to purified lecithins have proved unsuccessful, (e g Plaut and Rudy, 1931-1932, brain lecithin, and Kimizuka, 1935, egg lecithin) It appears therefore that purified lecithins mixed with serum are weakly antigenic, and that different lecithins show differences of immunological character, but these differences are not sharp

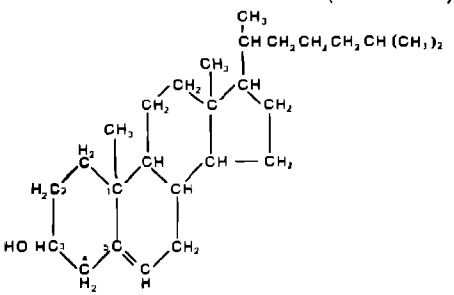
Ornstein (1926) also injected rabbits with cephalin (which differs from lecithin in the nature of the nitrogenous base) and cerebrosides (which contain a different base, sphingosin, galactose and only one fatty acid residue), and obtained sera which gave complement fixation specifically with the corresponding antigen No details of the preparation and purity of the lipin preparations are given

It is possible that the compounds involved when tissue extracts and impure preparations are used for immunization are more complex than lecithin Tropp and Baserga (1934) found that combination immunization with a polydiamino phosphatide obtained from spleen, produced sera which would fix complement in the presence of only 3 3γ of the lipin antigen This lipin is made up of three sphingomyelin molecules, each containing a different acid radicle—palmityl, stearyl and lignoceryl Sphingomyelin resembles lecithin in containing phosphoric acid and choline, but in place of glycerol it contains a base, sphingosine, to which is attached a fatty acid radicle The antisera reacted with the polydiamino phosphatide from liver, but not with sphingomyelin, cerebron, lecithin, heart

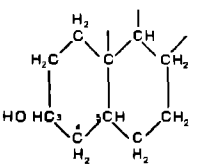
[1] It is necessary to add some protein solution, preferably pig serum, to lipin suspensions, before injection, in order to produce antibodies It should be understood that the procedure was used in the immunization procedures mentioned subsequently

extract or cholesterol Tropp and Baserga failed to obtain active sera by combined immunization with sphingomyelin or cerebron, but the number of rabbits used was small

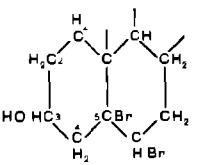
Sachs and Klopstock (1925) had demonstrated the antigenic power of cholesterol, Weil and Besser (1931, 1932) and Berger and Scholer



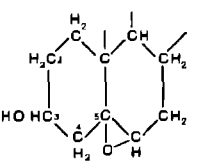
Cholesterol



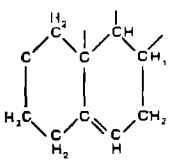
Dihydrocholesterol



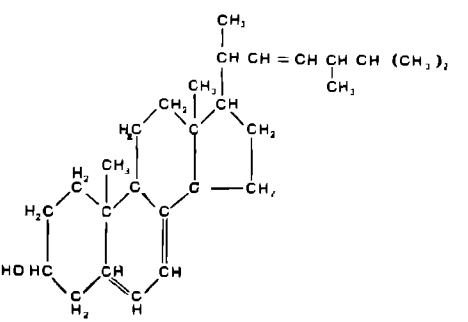
Dibromcholesterol



Oxycholesterol



Cholestan



Ergosterol

FIG XVI

(1932) compared the immunological properties of various sterols They found that cholesterol, dihydrocholesterol, oxycholesterol, and ergosterol produced active sera which gave complement fixation but no flocculation Cross-reactions of varying intensity were found, but stronger reactions occurred in all cases with the homologous antigen Various other sterols and sterol derivatives were tested against these

antisera. Antiergosterol serum gave no reaction with irradiated ergosterol from which the unaltered ergosterol had been removed, but gave reactions with sitosterol Immunological differences were demonstrated between cholesterol and pseudocholesterol (in which the double bond occupies a different position), cholestan, and cholesterol esters showing the importance of the presence and position of the —OH group , also between cholesterol and dihydrocholesterol, cholesterol dibromide and oxide, showing the importance of the double bond [1] Weil and Besser (1932) found that cholesterol oxide, dibromide, and esters were not antigenic Berger (1933) immunized with cholesterol benzoate which had been attached to protein The rabbit serum produced gave no reaction with cholesterol nor with cholesterol benzoate attached to any protein other than that used for immunization The failure to react with cholesterol might be expected as the —OH group is blocked The immunological behaviour of sterols, therefore, is related to the chemical structure in accordance with the instances already discussed

The whole theory of the formation of antibodies to simple lipins (purified lecithin, cephalin and cholesterol) has been challenged by Wadsworth, Maltaner and Maltaner (1934, 1935, a and b) They maintain that the reactions obtained are not specific and are due to anticomplementary action of the sera obtained and of the lipins This may well be the case with the purified phosphatides Hahn and Hazeto (1936), however, maintain that the complement fixation and flocculation obtained with cholesterol and the corresponding antiserum is specific , that is, it does not occur with other lipins, and cannot therefore be due to anticomplementary action

Antisera to organ emulsions react with alcohol extracts of organs, and similar antisera can be produced by the injection of alcohol extracts without the addition of protein , it is therefore generally considered that lipins are acting an antigens in these instances with the adjuvant action of traces of protein in the extracts However, these organ emulsions contain the debris of various cells and tissues, and can give little information on the relation of specificity to any particular constituent More satisfactory results are obtained with red blood corpuscles Haemolytic sera can be produced both by a protein fraction, and by an alcohol- and ether-soluble fraction which is generally considered to be a lipin, but, presumably, contains traces of protein These " lipin " antigens differ in their specific behaviour from the various protein antigens Instead of a gradual differentiation of immunological character from one species to those distantly related, due presumably to gradual changes of the complex protein molecule, sharp differences occur in related or even in the same species, and similarities in distantly related species (Landsteiner and van der Scheer, 1925) The Forssman antigen is the extreme instance of this This suggests that the specificity of these " lipin " antigens is not determined by differences such as those considered in dealing with

[1] The structural formulae given in Fig XVI are based on a paper by Heilbron, Simpson and Spring (1933)

natural protein antigens, but by complete differences in the determinant molecules Similar sharp immunological differences are found when the specificity is determined by carbohydrate fractions, as, for example, the differences between the types of pneumococci Of recent years evidence has accumulated which suggests that the active parts of these antigens actually are polysaccharides The active fraction of the Forssman antigen, from horse kidney, prepared by Landsteiner and Levene (1925, 1927, b and c) appeared to contain a polysaccharide Antisera to *B dysenteriae* (Shiga) haemolyse red blood corpuscles which contain the Forssman antigen Haemolysis of sheep's red corpuscles by such sera is inhibited by the carefully purified polysaccharide obtained by Morgan (1931) from smooth strains of Shiga's bacillus, presumably because the polysaccharide combines specifically with the haemolysin Meyer and Morgan (1935) have shown that, when the ability of this polysaccharide to form a precipitate with anti-Shiga serum is reduced by various treatments, the ability to inhibit haemolysin is reduced to a parallel degree If may be inferred that the antigen in Shiga's bacillus, which gives rise to the haemolysin, is this polysaccharide and that the active part of the Forsmann antigen is also a similar polysaccharide.

Hallauer (1934) extracted substances from human red blood corpuscles of group A and B which inhibited iso-agglutination by α and β sera respectively in quantities of the order of 1γ The A substance, in quantities of the order of 0 05 γ, inhibited haemolysis of sheep's red corpuscles by the serum of rabbits immunized with human A corpuscles The similar substance from O corpuscles did not inhibit iso-agglutination of either A or B corpuscles After hydrolysis these substances reduced Fehling's solution vigorously They contained about 7 per cent of nitrogen but no protein

Urine also contains group-specific substances These have been purified by Freudenberg and Eichel (1934, 1935) The composition and activity of that from "A" urine is given in Table XV A preparation was made by Brahn, Schiff and Weinmann (1932) from commercial pepsin, which inhibited iso-agglutination of group A cells by α-antibody, and haemolysis of sheep's red corpuscles by anti-A rabbit serum It contained carbohydrate and nitrogen The results of more complete examination of this substance by Landsteiner and Chase (1936) and of a similar substance from horse saliva by Landsteiner (1936, b) are given in Table XV

Somewhat analogous to the haemolysis of sheep cells by anti-Shiga serum and the inhibition of this haemolysis by Shiga polysaccharide is the inhibition by acetylated Type I S S S of the agglutination of Group A human red cells by Group B human serum, and of the haemolysis of sheep's red cells by anti-A rabbit serum (Witebski, Neter and Sobotka, 1935)

It is significant that such of these substances as have been fully examined contain an acetylated hexosamine and galactose Removal of the acetyl abolishes the specific activity of the A substance from

urine (Freudenberg and Eichel, 1935) and greatly reduces that of S S S. I However, not only the group A substances and the substances which give cross-reactions with the closely related Forsmann antigen, but also group B and O substances contain acetylated hexosamine and galactose The specific differences between these polysaccharides must be due to some other part of their structure

Jorpes and Norlin (1933) prepared a substance from urine which apparently was a protein This inhibited iso-agglutination, but far larger amounts of this preparation than of the polysaccharides described were required to produce this effect

Also a highly reactive polysaccharide is contained in the lipin fraction of the tubercle bacillus (see Table XV), on which part of the immunological activity of the bacillus depends

E Cross-Reactions

When an animal is immunized with a mixture of molecules of different species its serum may contain distinct antibodies to different antigenic molecules contained in the complex mixture substance When one of these antibodies is removed by adsorption with its appropriate antigen, the other antibodies are left unaffected When what is apparently a single molecular species is used for immunization the antiserum produce may react with substances which are not identical with that used for immunization In precipitin reactions, such heterologous antigens will remove part, but usually not all of the antibody to the homologous antigen (Table XVI) In most of the instances studied the amount of antibody left, after

TABLE XVI

Amount of precipitate formed by an antiserum with homologous and heterologous antigens (*Landsteiner and van der Scheer*, 1936)

Antiserum to m-amino-benzene-sulphonic acid coupled to horse serum protein

	Position of substituents		
Antigens made from chicken serum protein and—↓ (*Concentration* 0 01 *per cent*)	*Ortho* 	*Meta* 	*Para* 
Amino-benzene-sulphonic acid R = SO_3H	++±	+++	±
Amino-benzene-arsinic acid R = $As_2O_3H_2$	O	+	O
Amino-benzoic acid R = COOH	O	+	O

absorption by heterologous antigen, has been measured or recorded by only semi-quantitative methods, which may give confusing results One system in which quantitative methods were used was studied by Heidelberger and Kendall (1934) The antisera obtained by immunising rabbits with the highly coloured antigen R salt-azo-benzidine-azo-egg-albumin (apparently free from uncombined egg albumin) formed a precipitate with egg albumin In some instances egg albumin would not precipitate all the antibody to the dye antigen (e g only 56 per cent) This is the usual type of experience when such antigens are used But one antiserum was studied from which egg albumin, if added in sufficient amount, would precipitate all the antibody It is highly probable that in all these cross reactions in which a single molecular species is used as antigen, some of the antibody to homologous antigen is removed when an antiserum is absorbed or precipitated by a heterologous antigen that gives a visible reaction with it, and that, in those instances in which the amount of antibody to the homologous antigen is reported to be unaffected the methods of estimation or of recording the residual antibody have not been sufficiently accurate to show the reduction [1]

A special study of cross reactions has been made by Landsteiner and van der Scheer (1936) using several synthetic antigens The chief points in their work are illustrated by Tables XVI, XVII, XVIII Chicken stromata, coupled with the determinant groups specified, were used as convenient insoluble protein compounds for absorbing antibodies

TABLE XVII

Effect of absorbing a serum with heterologous antigens (Landsteiner and van der Scheer, 1936)

Antiserum to m-amino-benzene-sulphonic acid coupled to horse serum proteins This antiserum absorbed with antigens made with stromata of chicken red corpuscles Readings after 1 hour at room temperature (first line) and after standing overnight in ice box (second line)

Serum absorbed with stromata coupled with ↓	*Precipitate with test antigens made with chicken serum proteins and*			
	o-amino-benzene-sulphonic acid	*m-amino-benzene-sulphonic acid*	*m-amino-benzene-arsinic acid*	*m-amino-benzoic acid*
o-amino-benzene-sulphonic acid	O O	++± +++±	± ±	+ +
m-amino-benzene-arsinic acid	+± ++	+++ * ++++	O O	+ +±
m-amino-benzoic acid	+± ++	+++ * ++++	± ±	O f tr
Unabsorbed	++ +++	++++± * ++++	+ ++	+± ++±

[1] For example the results marked with an asterisk is Table XVII

It appears that one heterologous antigen, e g , *m*-amino-benzene-arsinic acid antigen, reduces the amount of antibody to the homologous antigen and largely removes the antibody that will precipitate with another heterologous antigen Table XVIII shows that a heterologous simple haptene will inhibit precipitate formation with heterologous antigen, but will inhibit precipitate formation with homologous antigen to a considerably less degree The concentrations of simple haptenes required to inhibit precipitate formation with heterologous antigen is considerably lower than those required to inhibit precipitate formation with homologous antigen

TABLE XVIII

Inhibition of reaction with homologous and heterologous antigens by homologous and heterologous simple haptenes (Landsteiner and van der Scheer, 1936)

Antiserum to m-amino-benzene-sulphonic acid coupled to horse serum

Antigens made from chicken serum protein and →	*m-amino-benzene-sulphonic-acid (Homologous Antigen)*				*m-amino-benzene arsinic acid (Heterologous Antigen)*			*m-amino-benzoic acid (Heterologous Antigen)*		
Haptene final concentration	0 025M				0 0035M			0 0035M		
Haptene	2	3	4	Control	2	3	Control	2	4	Control
Precipitate in 20 minutes	O	++	+±	++	O	+	+	O	±	+
Precipitate in 24 hours	+±	+++±	+++	+++±	±	+±	++	+	+±	++

Haptene 2 m-amino-benzene-sulphonic acid (homologous haptene)
3 m-amino-benzene-arsinic acid
4 m-amino-benzoic acid

Further examples of the inhibition of reactions of antisera with homologous and heterologous haptenes are found in the work of Avery, Goebel and Babers (1932) on glucosides (Table XII) and the more complicated work with disaccharides (Table XIII) of Goebel, Avery and Babers (1934) It might be suggested that these more complicated results might be the result of the presence in the serum of different antibodies to different parts of the determinant disaccharide group Thus the maltoside antiserum might contain separate antibodies to the terminal α-glucoside and the sub-terminal β-glucoside, and the cross reaction of the antiserum with the cellobiose antigen be a reaction of the β-glucoside antibody with this β-glucoside (in common) and possibly of the antibody to the α-glucoside with part of the terminal glucose If this were the case neither α- nor β-glucoside haptenes should wholly inhibit the precipitation of this serum with cellobiose , the β-glucoside haptene should inhibit only the reaction with the one antibody and the

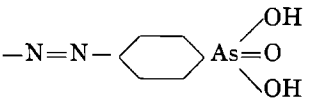
α-glucoside that with the second, neither should inhibit the reaction completely, as actually occurs (Table XIII) Also the β-glucoside haptene should inhibit the reaction of the one antibody and the α-glucoside haptene the reaction of the second with the *maltose* antigen. So that one or other or both these glucosides should considerably reduce the amount of precipitate formed by the antiserum and the maltose antigen This apparently does not happen

Again, as Landsteiner and van der Scheer (1936) point out, the antiserum to *m*-amino-benzene sulphonic acid might be supposed to contain antibodies (*a*) to an aromatic sulphonic acid and (*b*) to a benzene ring substituted by an acid in the meta position On this supposition it would be the antibody (*b*) that would react with both *m*-amino-benzene-arsinic acid and *m*-amino-benzoic acid antigens Absorbing the antiserum with *m*-amino-benzene-arsinic acid stromata should completely remove the antibody that forms a precipitate with *m*-amino-benzoic acid antigen, actually this is not the case (Table XVII)

The experiments quoted (Table XVII) show that the reaction of antiserum with heterologous haptene is more readily inhibited than that with homologous antigen Antibody is less firmly combined with heterologous antigen than that with homologous antigen and is therefore more readily replaced by haptene Such weaker combination is also suggested by the cross reaction of antiserum to R salt-azo-benzidine-egg-azo-albumin with native egg albumin Much more egg albumin than azo-egg albumin was required to precipitate a given amount of antibody, and a certain amount of egg albumin was always left in solution We may therefore infer that antisera to a given determinant group do not contain distinct antibodies to different parts of the determinant group (this does not imply that the antibody molecules are all uniform) and that combination of antibody with a heterologous determinant group is less firm than with the homologous determinant group

Since a modified antigen will not, as a general rule, precipitate all the antibody precipitable by the homologous antigen, the antibody molecules in a serum cannot be uniform Further evidence of this lack of uniformity is given in Chapter V in the discussion on quantitative aspects Burnet (1934) has suggested that antibody molecules may contain receptor sites (a), (b), (c), (d) corresponding to separate antigenic groups, A, B, C, D of the homologous antigen, and that these may be differently distributed on the antibody molecules, e g. (aaa), (abcd), (acd), (bc) (bbd) Only those antibody molecules which contain the receptor sites (a) would be precipitated by a heterologous antigen which shared only the atomic group A with the homologous antigen The antibody molecules would combine more firmly with homologous than with heterologous antigen, owing to the larger number of bonds of union Heidelberger and Kendall (1934) stressed the importance of dominant antigenic groups in the antigen, which combine firmly with corresponding receptor sites on the antibody molecule, and minor antigenic groups,

which combine only weakly with the corresponding receptors Morgan (1936, b) has combined these two views, he suggests that, since dominant groups are more highly antigenic, all the antibody molecules contain receptors (a) for a dominant group A, while fewer antibody molecules contain receptors (b), (c), (d), for minor antigenic groups B, C, D Antibody molecules, according to this idea, would have receptors a, ab, abc, etc As the immunization of an animal was prolonged more and more receptor sites to the minor antigens would appear on the antibody molecules In consequence the range of cross reactions of the serum would be extended The strength of combination of a heterologous antigen with the antibody molecules would depend on the presence or absence of the firm union A—(a), also on the number of the weaker unions B—(b), C—(c), D—(d) However, this plan would not account for the failure of *m*-amino-benzene-arsinic acid stromata to remove all the antibody that reacts with *m*-amino-benzoic acid antigen And, as Morgan (private communication) points out, it is not legitimate to regard separate sub-groups of atoms in a small determinant group such as



in isolation, any modification produces a change in the adjacent sub-groups (this point is considered more fully in the next chapter) In the case with which Heidelberger and Kendall (1934) were dealing, the dominant and minor groups may be widely separated, and this concept of separate antigenic groups and receptors may be justifiable But, when the antigenic sub-groups are not thus separated, it seems more satisfactory to suppose that the antibody molecules in any antiserum differ in the completeness of the adaptation of their receptor sites to the whole of the homologous or heterologous determinant groups, however this adaptation may be expressed Those more completely adapted will combine more firmly with the homologous antigen or with a heterologous antigen having certain parts in common Without any assumptions as to the exact nature of this adaptation, they may be spoken of as " more avid " antibody molecules

So far this discussion has dealt with artificial antigens, it has also been supposed that natural antigens, consisting of a single molecular species, may give rise to two distinct types of antibody molecules (a) and (b), owing to presence in one molecule of two antigenic groups A and B Thus Hooker and Boyd (1934) consider that the precipitate formation by duck egg albumin and antiserum to hen egg albumin, is due to the presence in the serum of a distinct antibody (b) to a minor antigenic group B common to hen albumin (antigenic groups A and B) and duck albumin (antigenic groups C and B) In support of this they find that the ratio of hen albumin antiserum at optimum proportions (see Chapter V) is not affected by precipitating from the antiserum all the antibody that will react with duck

albumin The optimum proportions they considered to be determined by the major antigen-antibody reaction A—(a) However, as subsequent investigations (1936) show, duck albumin removes only about $\frac{1}{8}$ of the total antibody protein from an antiserum to hen albumin This is only just sufficient for its removal to be detectable by the optimum proportions method as used by Hooker and Boyd As already noted, when some antibody molecules are removed the remainder will not behave in the same way as the original serum in the optimal proportions reaction, owing to the non-uniformity of antibody molecules This non-uniformity is illustrated, in this instance, by the fact that precipitation with hen albumin is much delayed after the removal of the small amount of antibody that reacts with duck albumin If only a minor antibody (b) was removed the reaction of (a) with A should not be affected in this way, but this delay would be expected if the duck albumin removed the more avid antibody molecules

Meyer and Morgan (1935) also consider that the polysaccharide of *B dysenteriae* (Shiga) gives rise to two antibodies (a) and (b) corresponding to two antigenic groups A and B of which (a) combines and forms a precipitate with the polysaccharide, while (b) combines with group B and also causes haemolysis of sheep's red corpuscles The presence of two antibodies in the antiserum to *B dysenteriae* is suggested by the great difference of character between a precipitin reaction and haemolysis The supposition does not, however, seem necessary The disorganization caused by precipitation of one of the constituents of the surface of a red blood corpuscle may well cause haemolysis Haemolysins are known to form precipitates with extracts of red corpuscles (Eagle, 1935)

In neither of the instances considered can the formation of two distinct species of antibody molecules be excluded But the homologous and heterologous antigens, in the two instances considered, may differ as do synthetic antigens formed from *m*-amino-benzene-sulphonic acid and *o*-amino-benzene-sulphonic acid , and the antibody molecules in the antiserum to one antigen may differ in degree rather than in kind

This discussion has an important bearing on the mosaic conception of bacterial structure If a bacillus Q will remove part and only part of the antibody from an antiserum to bacillus P it does not follow that P contains two distinct antigenic molecules A and B, one of which it shares with Q It may be that P contains an antigenic molecule with two antigenic groupings, one of which is also contained in antigenic molecules of Q The antigenic molecule of P would be comparable to the azo-egg-albumin antigen of Heidelberger and Kendall (1934) Furth and Landsteiner (1929) found that a serum, which reacted with only one bacterial haptene (e g 8 of B paratyphosus B), precipitated other haptenes (II and 7) along with it, just as an anti-egg-albumin serum may precipitate the whole azo-egg-albumin molecule Alternatively, P may contain an antigenic group, A and Q a similar, but not identical, antigenic group

A^1,'A and A^1 differing as do *m*-amino-benzene-sulphonic acid and *o*-amino-benzene-sulphonic acid

It may be suggested, as a postulate, that the presence of wo separate antigenic substances A and B in a complex antigen (such as bacillus) can only be inferred (1) when A and B have been separated, and (2) when removal of the antibody to A, by precipitating with A, leaves the antibody to B unreduced, and vice versa

It is less difficult to account for the occurrence of cross-reactions than for the failure of cross-reactions Why, for example, when antisera to pneumococci Types II and III form precipitates with gum acacia and synthetic glucoside antigens owing to the common presence of glucuronic acid, should Type II antiserum not react with S S S III and vice versa The answer to this question requires a fuller knowledge of the composition of S S S II and structure of S S S III For although S S S III is known to be built up of a series of aldobionic acid (glucuronic acid—glucose) molecules, it is not known whether it consists of a chain of alternating glucose and glucuronic acid molecules or of a chain of glucose molecules with glucuronic acids attached

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CHAPTER IV

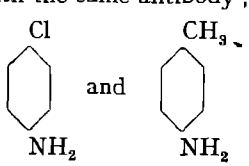
THE NATURE OF ANTIGEN-ANTIBODY REACTION FIRST STAGE COMBINATION

In the preceding chapter we have seen that proteins which contain certain determinant groups will react specifically with appropriate antibodies This might mean that the " determinant groups " conferred a specific ability to combine with the antibody, or a specific ability to form a precipitate after non-specific adsorption had occurred The inhibition of precipitation by simple haptenes, which themselves do not form a precipitate with the antibody, can be most readily explained by the first supposition The effect would resemble the competitive inhibition observed in enzyme reactions (Haldane, 1930, b) The simple haptene, present in excess, occupies the combining sites on the antibody and prevents its combination with antigen That such specific combination of simple haptenes with antibodies actually occurs has been demonstrated directly by Marrack and Smith (1932), and later by Haurowitz and Breinl (1933)

Since no further reaction, such as precipitation, occurs as a rule when a simple haptene combines with antibody, it is possible to distinguish two stages in an immunity reaction (1) specific combination of the " determinant groups " with antibody, (2) secondary reaction—precipitation, agglutination, &c Since the " determinant group " combines specifically with antibody, it is possible to study examples of specific combination in which the chemical structure of one of the partners to the combination—that is the " determinant group "—is known

A The Mode of Combination of Antibody and Antigen

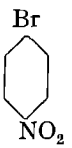
Considering first the stage of combination, we find that many of the " determinant groups " that combine with antibody are not characterized by any chemically reactive radicles On the other hand, we find that " determinant groups " of different chemical composition may be immunologically equivalent, which implies that they combine equally with the same antibody , for example,



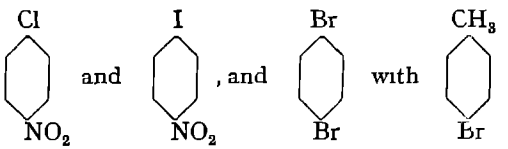
Such a result can only be ascribed to intermolecular forces and the specific character must be the result of (1) the presence of atoms or groups of atoms with appropriate fields of force in the determinant group and in the receptor sites of the antibody , (2) a proper spatial distribution such that corresponding active points of antigen and

antibody can come in apposition simultaneously, and (3)* the accessibility of the active points of the determinant group to the antibody owing to the absence of any large inactive group that would prevent this approach These are the same factors as determine the specific selection of molecules that are built into a crystal Erlenmeyer and Berger (1932, a) have drawn attention to the immunological equivalence of such atoms or radicles as are mutually replacable in crystals However the formation of mixed crystals is a severer test than immunological similarity In crystal building molecules have to fit, in dimensions and distribution of polar fields, in every dimension, also a foreign molecule B, in order to be built into a crystal of A has to compete for its place with molecules of A, while in the combination of determinant group and antibody only one surface need fit and there is no competition with other molecules whose fit is unquestioned Hence the occurrence of cross-reactions between antigens containing active groups which cannot replace each other in crystals A better comparison would be with adsorption on the surface of crystals, but specific adsorption to which comparison could be made, has been little studied

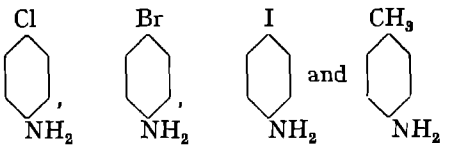
This thesis of Erlenmeyer and Berger is however illustrated in the following instance The halogens and $-CH_3$ are mutually replaceable in crystals, for example



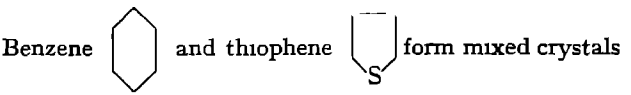
will form mixed crystals with



Immunologically the determinant groups



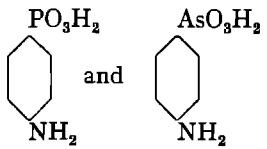
are practically equivalent (Landsteiner and van der Scheer, 1927) The NO_2 compound gives cross reactions but they are not so strong



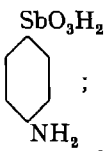
and there is considerable physical similarity between benzene, thiophene and furane

(Erlenmeyer and Leo, 1933) The immunological similarity of these three rings is shown in the work of Landsteiner and van der Scheer (1931, a) already quoted (p 75) in which thiophene appears more closely related to benzene than is furane , also by the similar behaviour of benzene, thiophene and furane found by Berger and Erlenmeyer (1933)

Erlenmeyer and Berger (1932, b) have also found that the " determinant groups "



were immunologically equivalent and different from

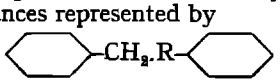


;

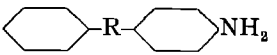
which is in accordance with the isomorphism of the salts of H_3PO_4 and H_3AsO_4 (e g Na_2HPO_4, $12H_2O$ and Na_2HAsO_4, $12H_2O$), and entire dissimilarity of H_3SbO_4

Haurowitz and Breinl (1933) also demonstrated the immunological difference between As and Sb, and between pentavalent and trivalent As Erlenmeyer and Berger (1933) found that reactions between *p*-amino-benzene-sulphonic acid ($NH_2 C_6H_4 SO_3H$) azo-protein and the corresponding antibody were inhibited almost as well by benzene-selenonic acid as by benzene-sulphonic acid This is in accordance with the isomorphism of sulphuric and selenic acids and their salts

Erlenmeyer and Berger (1932, a) have also stressed the importance of local similarities of electric field, resulting from equality of the number of valency electrons of atoms or pseudo-atoms in determining their mutual replaceability in crystals and their immunological similarity The term " pseudo-atom " has been applied to a group of atoms which can be considered to function as one atom This theory fits CH_3, Cl, Br and I, which are found approximately equivalent , also P and As, and S and Se The chief example cited by Erlenmeyer and Berger concerned the atom O and the pseudo-atoms CH_2 and NH_2 These are mutually replaceable in the crystals of the substances represented by



where R is O, CH_2 or NH In this instance the number of valency electrons is 6, e g in the pseudo-atom CH_2 there are 4 from the C atom and 2 from the two H atoms In CO the number of valency electrons is 10 (4 from the C atom and 6 from the O atom) The compound in which CO takes the place of R cannot replace the other three compounds in crystals Erlenmeyer and Berger considered that the compounds,



in which R was O, CH_2 or NH, were immunologically similar and sharply distinguished from the compound in which CO took the place of R However, as noted in Chapter III, Jacobs (1937) found no sharp distinction between these O, CH_2 and CO compounds It is probable that other factors, besides the local electric field at R, affect these serological reactions In the case of benzene and thiophene, in which the pseudo-atom —CH=CH— is equivalent to —S— it is necessary to neglect the 4 electrons concerned in the double bond as being concerned in the inner structure of the —CH=CH— pseudo-atom On this reasoning C=O should be equivalent to O In fact all groups of atoms which have the same number of free valencies must contain the same number of valency electrons, unless, in some of them, hydrogen has been substituted by a larger atom or group of atoms In which case, the steric effect of such a substituent may be expected to play a part in determining the possibility of mixed crystal formation and the immunological specificity

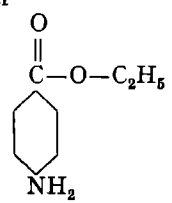
Moderate differences of distribution of electric charge in a determinant group seem to be compatible with immunological similarity, for

$$\underset{\text{Cl,}}{-0\ 21} \quad \underset{\text{Br}}{-0\ 19} \quad \text{and} \quad \underset{\text{I}}{-0\ 15}$$

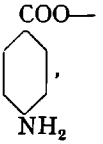
have, when attached to carbon, negative charges approximately equal to the decimals of an electron charge shown, while

$$\underset{-CH_3}{-0\ 12} + 0\ 12$$

is partly positive and partly negative Greater differences entirely alter the specificity Proteins coupled with diazotized *p*-aminobenzoic acid ethyl ester



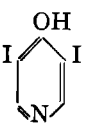
(which is not ionized) do not react with antisera to *p*-amino-benzoic acid, which is ionized as



and forms precipitates with antisera to the neutral antigens formed from aniline, *p*-toluidine and *p*-nitro-aniline On the other hand protein coupled with free *p* amino-benzoic acid does not give a precipitate with antisera to neutral antigens (Landsteiner and van der Scheer, 1927)

The introduction of polar groups into the less active parts of a determinant group affects the specificity Thus the inhibitory effects of 4-carbon dicarboxylic acids on the precipitate formed by *p*-succinanilic acid protein and the corresponding antibody is in the order $COOH\ CH_2\ CH_2\ COOH$ (succinic acid) $>COOH\ CH(OH)\ CH_2\ COOH > COOH\ CH(OH)\ CH(OH)\ COOH = COOH\ CH(NH_2)\ CH_2\ COOH$ (Landsteiner and van der Scheer, 1934) The effect of these substituents may be due both to their local effect on the electric field and to an electron shift along the carbon chain, which shows itself by changes produced in the terminal —COOH groups It is remarkable that Snapper and Grunbaum (1936) should have found that the acetic ester of diiodo-4-hydroxy-benzoic acid, $CH_3\ CO\ O\ C_6H_2I_2\ COOH$, should inhibit precipitate formation by iodo-proteins and the corresponding antisera For at the hydrogen ion concentrations involved, the —OH group of diiodotyrosine (which is the determinant group of iodo-proteins) is almost fully dissociated, as the pK of this group is 6 48 (Dalton *et al*, 1930) The failure of the ether, $CH_3\ CO\ C_6H_2I_2\ COOH$ to inhibit is to be expected The inhibitory effect of the pyridon compound

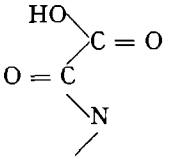
may be due to enolization to the form



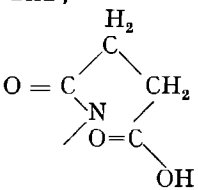
The ultraviolet absorption curve of the pyridon compound still shows some resemblance to that of phenol, whereas that of the methyl ether is wholly different , a difference of electron distribution in the two compounds is thus shown

The above examples illustrate the importance of the first criterion The second criterion is illustrated by the specific differences between *l*- and *d*-tartranilic acid antigens (p 79) Suppose that the active points of these antigens are the two —OH groups, the —COOH and the —CO—NH— groups, and that the antibody to the *d*- antigen has 4 receptors arranged, in a plane, to fit these four points of the *d*- antigen It will then be impossible for more than two of the active points of the *l*- antigen or three of the active points of the meso-antigen to be in contact with these receptors at the same time Hence the antiserum to the *d*- antigen forms some precipitate with the *m*- antigen but very little with the *l*- antigen

In the case of the long chain anilic acids (p 76) such as C_6H_5 NH CO $(CH_2)_4$ COOH the active points —NH CO and —COOH can be brought in contact with the receptors of the antibodies to antigens of other chain lengths owing to the flexibility of the chain Hence the cross-reactions found between, for example, adipanilic [C_6H_5 NH CO $(CH_2)_4$ COOH] antigen and suberanilic [C_6H_5 NH CO $(CH_2)_6$ COOH] antibody On the other hand, the receptors which are so placed as to fit oxanilic acid

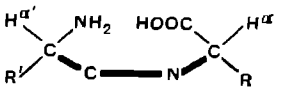


will not fit succinanilic acid ,



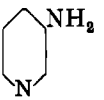
hence there are no cross-reactions between these compounds The fact that the reaction of succinanilic antigen with the corresponding antibody is inhibited by the *cis* compound, maleic acid, (p 80) and not by the trans compound fumaric acid, shows that the succinanilic acid takes the form shown above

The importance of the third criterion may first be illustrated by its effect in an enzyme action Dipeptidase (Bergman *et al* , 1935) appears to combine with and hydrolyse dipeptides in the flat hexagonal form , the enzyme fits to the upper surface of the hexagon

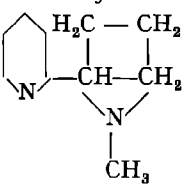


If one of the hydrogens α and α', which are above the plane of the hexagon, is replaced by CH_3 groups, hydrolysis of the enzyme is considerably slowed But if either is substituted by an *iso*butyl

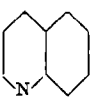
group [$-CH_2\,CH(CH_3)_2$] the action of the enzyme is prevented because the enzyme cannot approach near enough to the binding plane of the substrate There are not many unquestionable examples of a similar effect in antigen-antibody reactions One reason for this is that the determinant groups of synthetic antigens have mainly been aniline derivatives In these the hydrogen atoms or substituents for them lie in the plane of the ring Hence substituents, however large, need not interfere with the approach of the receptor site of the antibody to the face of the ring A striking instance of this is the inhibition of the reaction between β-amino-pyridine antigens



and the corresponding antibodies by nicotine

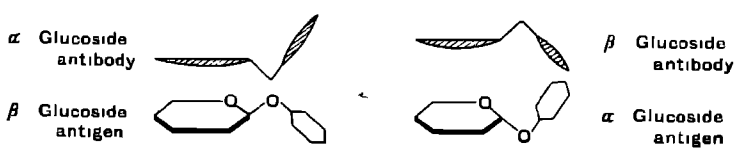


and by quinoline



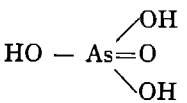
in spite of the large substituents (Berger and Erlenmeyer, 1935) The incomplete precipitation of the precipitin for S S S III by methylated S S S III may be due to obstruction by the inert groups introduced, and the effect of acetylation of glucose antigens (p 82) on their specificity to the obstruction of the approach of the anti-glucose antibody by the

introduced The specific difference between α and β-glucoside antigens (p 81) may also be attributed to obstruction Thus if the binding face of the glucosides is the upper face in the figure on p 81 the oxygen of the glucoside link will obstruct the approach of the α-glucoside antibody to the binding face of the β-glucoside antigen, and the phenol ring will obstruct the approach of the β-glucoside antibody to the binding face of the α-glucoside

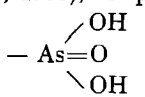


Benzoylation and subsequent attachment of protein to S S S' III does not affect its reaction with Type III serum (Avery and Goebel, 1931) This suggests that the benzoyl group is attached on the opposite face of the sugar ring to that which is adapted to the antibody receptors Similarly the methyl groups, 2 per equivalent, introduced into S S S I (Chow and Goebel, 1935) may not be on the binding face It is not necessary that the substitution of H by CH_3 on the binding face should prevent reaction, but it would probably weaken it

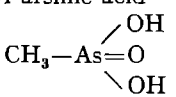
Specific combination of a determinant group with antibody involves more than a group of a few atoms even though such a subgroup may, to a large extent, determine the specificity Thus arsenic acid



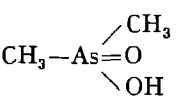
has only a very weak inhibitory action on precipitate formation by amino-benzene-arsinic acid antigens and antisera (Landsteiner, 1920 , Haurowitz and Breinl, 1933), in spite of the



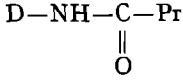
in common , while methyl arsinic acid



and cacodylic acid

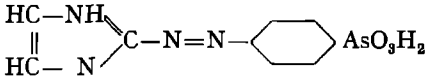


(Landsteiner, 1920) have no inhibitory action It is even possible that the inhibitory action of arsenic acid is non-specific, as Landsteiner found that it also has a slight inhibitory action on the benzene-sulphonic acid antigen-antibody system According to Haurowitz and Breinl (1933), a M/160 solution of *p*-amino-benzene-arsinic acid has considerably more inhibitory effect on the benzene-arsinic acid system than has M/5 arsinic acid When an azo-dye is formed, as by coupling with tyrosine, the inhibitory effect is much enhanced (see p 73 and Erlenmeyer and Berger, 1933, for examples) Other substances in place of this azo link may also enhance the inhibitory effect For example, the *p*-amino-benzene-sulphonic acid system is inhibited even more effectively by aceto-phenone-sulphonic acid $CH_3\,CO\,C_6H_4\,SO_3H$ than by the tyrosine-coupled compound (Erlenmeyer and Berger, 1933) On the other hand, synthetic antigens in which the determinant group (D) is joined to protein (Pr) by a ureido link

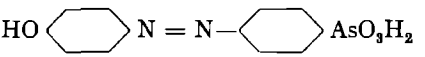


are not equivalent to those in which D is attached by an azo link (D—N = N—Pr) (Mutsaars and Grégoire, 1936)

Hooker and Boyd (1933) have found that iminazol-azo-benzene-arsinic acid

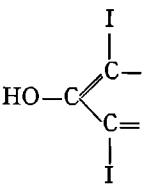


is about three times more effective than phenol-azo-benzene-arsinic acid

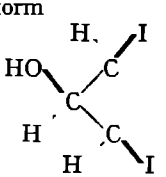


in inhibiting precipitate formation by casein coupled with *p*-amino-benzene-arsinic acid and an antiserum to egg albumin coupled with this arsinic acid, whereas the iminazol compound is 10 times more effective than the phenol compound in inhibiting precipitate formation by the casein compound and an antiserum to gelatin coupled with this arsinic acid It appears that the antiserum to the egg-albumin compound contains antibodies to the determinant group attached to tyrosine (a phenol derivative) and to histidine (an iminazol derivative) whereas the antiserum to the gelatin (which contains no tyrosine) compound contains antibodies to the histidine derivative only According to this supposition combination with the receptor site of the antibody is affected even by the structure on the far side of the azo link to the essential $-AsO_3H_2$ sub-group

The succinanilic acid system is inhibited by approximately 0 02 M succinanilic acid to the same degree as by 0 13 M succinic acid (Landsteiner and van der Scheer, 1934, a) Similar examples of the importance of the whole determinant group are found in the work of Landsteiner and van der Scheer (1932, 1934, b) on polypeptide antigens, and of Avery and Goebel (1929) on glucoside antigens Again Snapper and Grunbaum (1936) found that the iodo-protein system was inhibited by the atomic arrangement



when the three carbon atoms formed part of an aromatic ring But the aliphatic compound CH_3 CHI CH(OH) CHI CH_3 did not inhibit, although it can take the form



in which the spacing of the essential —OH and I atoms differ little from that in the aromatic compounds

Such effects of the whole of the determinant group in inhibition cannot be ascribed to the presence in the antisera of separate antibodies to different parts of the determinant group For example, suppose that the succinanilic acid antigen gave rise to separate antibodies strictly specific for the —NH CO CH_2— and —COOH parts of the antigen , then the first should not be inhibited by succinic acid COOH CH_2 CH_2 COOH in any concentration Actually the succinanilic acid system is inhibited by succinic acid, though in relatively high concentrations

The effect of certain sub-groups both in producing antibodies and in determining specificity may vary greatly The peculiar efficacy of arsinic acids in producing strong antisera has led to the constant use of benzene-arsinic-acid antigens for work with synthetic antigen-antibody systems Antisera to *p*-amino-benzene-arsinic acid antigens give precipitates with test antigens made from any benzene-arsinic acid Amino-sulphonic and amino-benzoic acids form less effective antigens which are less specific for the acid group , while antigens derived from aniline, toluidine and mono-halogen anilines give rise to weak sera which cross-react with other antigens containing a similar characterless group in the same position in the benzene ring Since such sub-groups as arsinic acid (which, using a term introduced by Heidelberger and Kendall, 1934, may be called "dominant" sub-groups) have a predominant effect in determining specificity, the firmness of the attachment of determinant group to the receptor sites of the antibody depends mainly but not wholly on these sub-groups

The receptor sites of the antibodies should therefore be considered to be adapted to receive a considerable area of the determinant group The effect of changes such as the introduction of, for example, a Cl atom into the benzene ring of a determinant group may be ascribed both to a local effect on the electric field, affecting the local adaptation to the receptor site, and to an electron drift in the benzene ring, which affects the adaptation of the whole ring or of any dominant group attached to it,[1] to the receptor site

Even the nature of the protein combined with the determinant groups in a synthetic antigen affects the amount of precipitate obtained with an antiserum to the determinant group A serum obtained by immunizing with a synthetic antigen A made from protein A (e g horse serum globulin-azo-benzene-arsinic acid) will give more precipitate with an antigen B made from protein B, which is closely related to A (e g dog serum globulin-azo-benzene-arsinic acid), than with an antigen C, made from a protein not closely related to A (e g gliadin-azo-benzene-arsinic acid), although the antiserum forms no precipitate with protein B Also the presence of a minimum

[1] Distant effects resulting from such substituents are shown by the influence of one substituent in a benzene ring on the site of entry of further substituents, also by the effect on dissociation constants of acids

number of determinant groups in the test antigen is necessary for precipitate formation Antibodies to *p*-amino-benzene-arsinic acid will not form precipitates with test antigens in which the As/N ratio is below 0 065 (Hooker and Boyd, 1932) This failure to form a precipitate may be due in part to failure in the second stage, discussed in the next chapter, but is due at least in part to the weakness of combination of antigen and antibody. In the case of antigen C the spacing of the determinant groups on the antigen molecule is different from that on the antigen molecules A and B Hence the determinant groups on antigen C cannot all simultaneously come in contact with the receptor sites on the antibody molecules, which are spaced to receive the determinant groups of A Both in such a case and when the number of determinant groups on the antigen is small the weakness of combination is due to the fewness of the determinant groups that come in apposition to the antibody receptor sites

The evidence, therefore, indicates that the specific combination of these determinant groups with antibodies is determined by the same factors which determine the specific binding of molecules together in the form of crystals, that is, the shapes of the molecules and the spatial distribution and strength of the polar forces It is then necessary to consider the nature of the change in serum globulin that converts it into antibodies with the power of combining specifically with determinant groups It is difficult to imagine the possibility of the attachment to serum globulin of prosthetic groups sufficiently varied to suit specifically all the determinant groups that have been used On the other hand, the surface area of a serum globulin molecule is large enough to accommodate some 150 benzene rings This surface must present a mosaic of groups of varying degrees of polarity It is extremely probable that some areas should occur which are specially suitable for binding particular molecules, this would account for the adsorption of dyes by normal serum proteins It is conceivable that slight alterations in the structure of globulin molecules might give rise to areas with a specific affinity for certain determinant groups One can compare the effect of the simple change from diamond to graphite packing of carbon atoms on the adsorption of methylene blue and succinic acid The formation of antibodies from serum globulin may be based on such a rearrangement

We may therefore suppose that there are certain receptor sites on the molecules of antibody globulins It is quite intelligible that the presence of such modifications should make no difference to the globulin detectable by ordinary means, or in some cases produce slight differences in its non-specific adsorption as already indicated

In some cases there may be some indication of the nature of the groups involved at these receptor sites The importance of the uronic acids of the pneumococcal and some other polysaccharides was noted in the last chapter Chow and Goebel (1935) have suggested that the combination between these polysaccharides and

the corresponding antibodies may be largely effected by attraction between the carboxyl groups of the uronic acids (which at the hydrogen ion concentrations involved are ionized as COO^- and are negatively charged) and the NH_2 groups of the antibody proteins (which also are ionized and positively charged) They found that, if the NH_2 groups of antibody were acetylated ($NH\ CO\ CH_3$), the antibody lost to a large extent its ability to precipitate with the corresponding polysaccharide However, precipitates were still formed with higher concentrations of the polysaccharide (down to 1/8000), so that other groups of the antibody must be involved in the reaction Treatment with formalin, which also changes the NH_2 groups, completely abolished precipitate formation with polysaccharide The ability to form a precipitate was to some extent restored after bringing the pH to 4 0 and standing at 0° C for several days, a treatment which probably restores the NH_2 groups It is likely, however, that the changes in the free NH_2 groups of proteins are accompanied by some disorganization of the protein molecule, and the loss of power to form precipitates with the homologous polysaccharide may be only indirectly due to the change in the NH_2 group The evidence is, therefore, not conclusive

Supposing that NH_2 groups are essential, the proportion of the total NH_2 groups of antibody protein involved is small The maximum ratio of S S S III to protein in the precipitate, formed with antiserum is about 1/20 (Heidelberger and Kendall, 1935, b), that is, one gram-equivalent of S S S III to 7,000 grams of protein, which contains some 7 NH_2 groups Even if the antibody contains extra NH_2 groups to combine with the COOH groups of S S S III, this would involve only a 14 per cent increase in the total NH_2 content of the protein

Specific combination must recall the combination of enzyme and substrate, and it is necessary to bear in mind the possibility of combination also occurring by other means, such as Schiff's reaction between $-NH_2$ groups and aldehyde groups, or the formation of salts (Waldschmidt-Leitz, 1932) Brunius and von Gard (quoted by Euler and Brunius, 1931) found that the binding of antibody by red blood corpuscles was not affected by nitrous acid, which converts $-NH_2$ into $-OH$ groups Schiff's reaction is therefore not involved in this process

Diphtheria antitoxin, adsorbed on charcoal (Eisler, 1923) or collodion (Freund, 1932), will not neutralize toxin It appears that the "adsorbing site" of the antitoxin is involved in adsorption on the charcoal or collodion In other instances, e g haemagglutinin (Eisler, 1924), this is not the case The adsorbing sites of a globulin acting as an antibody appear different to those by which it is bound when acting as an antigen Eisler (1920) found that antitoxin was still precipitated by a precipitin after binding toxin, Smith and Marrack (1930, a) found that diphtheria antitoxin precipitated by an anti-horse-globulin precipitin would still bind toxin, and that toxin-antitoxin floccules, resuspended, were flocculated by precipitin The sites

on the antitoxin molecule which bind toxin are therefore not the same as those at which it is attached to precipitin

There is also the possibility that the immunological characters of particulate antigens may be determined, not by the structure of certain molecules in them, but by the pattern of different molecules on their surfaces If this were the case the whole antigen would have immunological characteristics not shown by separate substances extracted from it

B Effect of Varied Conditions on Combination of Antibody and Antigen

Actual combination of simple haptene and antibody has been demonstrated by compensation dialysis methods by Marrack and Smith (1932) and Haurowitz and Breinl (1933) The amount of haptene bound by antibody was small In the experiments of Marrack and Smith, bound haptene was never more than 66 per cent of the total [1] One c c of a solution of antibody to *p*-amino-benzene-arsinic acid, which would combine with 0 21 mille-equivalents of arsenic contained in protein antigen, only bound 0 0355 mille-equivalents of arsenic in the form of simple haptene The dissociation constant of the haptene-antibody compound is therefore high The greater combination between antibody and protein antigen may be due to two causes First the protein antigen molecule has numerous determinant groups, which act as bonds of union with the reception sites on the antibody molecule The resulting combination is firmer than that between the antibody molecule and the haptene molecule, in which only one or two such bonds are involved In the second place, in the case of full antigen and antibody, an insoluble compound is formed by the combination, this will precipitate out Progressively more antigen-antibody compound will form and precipitate, and the concentration of antibody and antigen will fall until eventually they are in equilibrium with the saturation concentration of the antigen-antibody compound If the solubility of the compound is decreased, for example by a secondary reaction between antigen and antibody, more free antibody and antigen will combine and precipitate, although the dissociation constant of the compound may be unaltered

With the ordinary methods of investigation it is difficult to distinguish effects of, for example, pH and salt concentration on the combination of antigen and antibody, from effects on the solubility of the antigen-antibody compound In order to study the effects on the combination of antibody and antigen it is usual to suspend a particulate antigen in the antibody solution, centrifuge this antigen off and test the supernatant fluid for residual antibody It is then

[1] Haurowitz and Breinl corrected for non-specific adsorption of the haptene by serum proteins by dialysing with normal serum as the external solution and anti-serum as the internal solution As, however, anti-serum may contain considerably more protein than normal serum this correction may be inadequate

usually impossible to distinguish the effects on the dissociation constant of antigen and antibody from the possible effects on the solubility of the antigen-antibody compound resulting from secondary reactions

1 *Effect of pH and Salt Concentration*

Coulter (1920–1, b) found the binding of haemolysin was maximum at pH 5 3, that is, near the isoelectric point of globulin In the absence of salt this effect of pH was much more conspicuous, as the amount combined was negligible at pH 7 but at pH 5 3 was as high as that bound when salt was present Similar results were obtained by Euler and Brunius (1931) working on the combination of haemolysin with the stromata of red corpuscles (Fig XVII) Coulter found that the combination was fully reversible , the final results were the

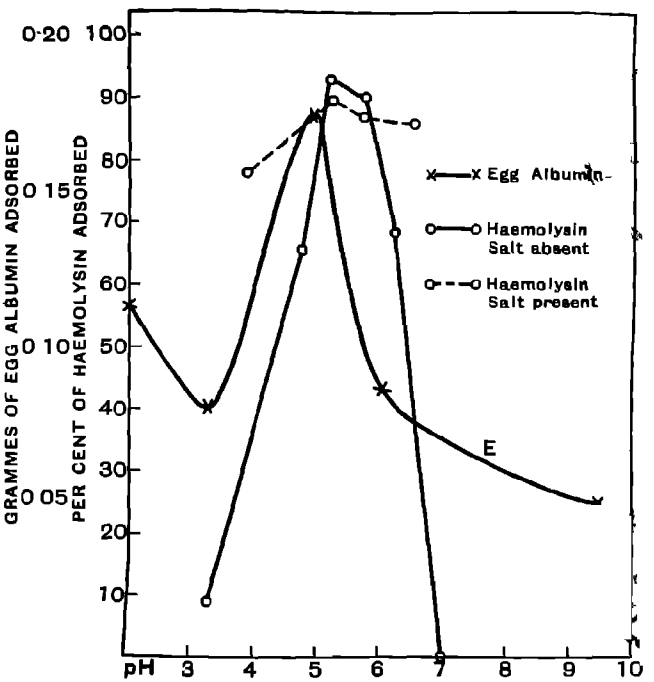


Fig XVII Adsorption of egg albumin by collodion particles (Hitchcock, 1926), and of haemolysin by stromata in the absence and in the presence of salt (Coulter, 1920–1, b).

same, whether the corpuscles were first fully sensitized and then suspended in solutions at the various pH levels, or suspended from the first solutions of haemolysin at the various pH levels. Euler and

Brinius found that the combination of haemolysin with lipin extracted from red corpuscles was also maximum at a pH just above 5, but that the reduction of the combination at other pH levels in the absence of salt was not so striking as that found with stromata This they attributed to the much lower degree of reversibility of the combination of lipin with haemolysin The symmetrical nature of the curve on each side of the maximum shows that the combination does not depend on the degree of ionization of any groups whose degree of ionization changes within the pH range studied The curve bears a close resemblance to that for the adsorption of egg albumin by collodion

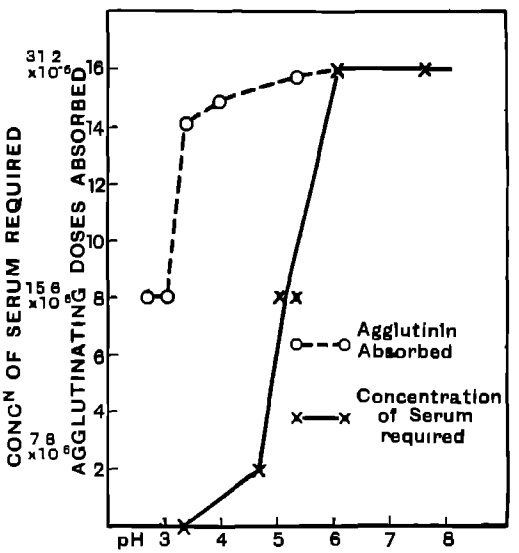


FIG XVIII Effect of pH on the amount of agglutinin absorbed by typhoid bacilli and on the minimum concentration of immune serum required to produce agglutination No agglutination occurred at pH under 3 3 (De Kruif and Northrop, 1922–3, a)

(Chap I) , as in this instance, the maximum adsorption at the isoelectric point of globulin may be due to the minimum ionization at this point and comparable to the greater adsorption of acids when in the un-ionized state found by Phelps and Peters (1929)

The relation to pH of the adsorption of agglutinin by typhoid bacilli found by de Kruif and Northrop (1922–3, a) is very different It cannot be concluded from their results that the dissociation constant of the antigen-antibody combination was completely unaffected by pH from 4 to 7 5, as nearly all the agglutinin was absorbed in all the experiments in this range But below this range a considerable reduction in the amount bound took place (Fig XVIII) This may mean that the combination depends on the degree of ionization of the

—COOH groups A comparison may be made with the combination of the enzyme saccharase with cane sugar , the dissociation constant of this compound is unaffected by pH about 5 6, but is doubled at pH 2 5 (Haldane, 1930, a)

Schmidt (1930, b) considered that diphtheria toxin and antitoxin did not combine in the absence of salt, because he could not obtain the Danysz phenomenon by adding toxin, in separate portions, to antitoxin in the absence of salt As, however, the Danysz phenomenon depends on further irreversible changes, its absence cannot be taken as evidence that the toxin and antitoxin did not combine, but merely that the further change did not occur

Landsteiner and Welecki (1911) found that the combination of haemolysin with red blood corpuscles was considerably reduced by hypertonic (1 N) sodium chloride solution This however, does not agree with the findings of Angerer (1909)

Duncan (1934) found that variations of salt concentration had little effect on the combination of *o*-agglutinins with smooth non-flagellated strains of intestinal bacteria Thus when an excessive amount of agglutinin was added to a suspension of bacteria approximately 90 per cent was bound with 1 35 N sodium chloride concentration, 76 per cent with 0 14 N sodium chloride and 72 per cent with 0 007 N sodium chloride On the other hand when the amount of agglutinin added was low, approximately 78 per cent was bound at 1 25 N sodium chloride and nearly 100 per cent at 0 15 N and 0 007 N With flagellar agglutinin the effect of salt concentration was entirely different (Duncan, 1937) The agglutinin combined rose from approximately 50 per cent at 1 2 N to 100 per cent at 0 001 N, and fell to 75 per cent in distilled water

Heidelberger, Kendall and Teorell (1936) found that in high salt concentrations a given amount of pneumococcal polysaccharide formed less precipitate from a given amount of antiserum than in 0 15 N salt This was, in part, due to decreased combination, as free antibody could be obtained from the supernatant fluid

This reduced combination in high salt concentration may be attributed to the atmosphere of salt ions round the oppositely charged polar groups of antigen and antibody reducing the attraction of these for one another The effect of salt concentration on the physical properties of polysaccharides (Heidelberger and Kendall, 1932, a and c) must also be taken into account

2 *Effect of Temperature*

The combination of haemolysin with red blood corpuscles at varying temperatures (Table XIX) was studied by Cromwell (1923) The temperature at which maximum combination occurred varied with different sera from 15° to 40° C This suggests that a secondary process may occur at higher temperatures, fixing the antibody more firmly and to some extent counteracting the increase with rising temperature of the dissociation constant of the compound first formed

TABLE XIX

The effect of temperature on the combination of haemolysin with red blood corpuscles (*Cromwell*, 1923)

		Number of haemolytic units added		
		100	200	800
Number of haemolytic units adsorbed at—	0° C	92	180	580
	15° C	95	187	600
	25° C	95	187	600
	37° C	90	180	575
	40° C	88	175	575
	45° C	83	163	500
	50° C	75	155	450
	55° C		120	400
	60° C		75	300

3. *Rate of Combination*

Cromwell (1923) found that the amount of haemolysin fixed by red blood corpuscles did not increase after 15 minutes Dreyer and Douglas (1910) found that the amount of agglutinin fixed by bacteria increased slightly up to 4 hours, the greater part of the agglutinin was taken up within the first 15 minutes This rapid combination of antigen and antibody has been cited as evidence that the fixation is not an adsorption There is, however, no reason why adsorption should not be as rapid as any other combination in which it is necessary that molecules should be suitably oriented in relation to each other before combination occurs The slow adsorption by charcoal is due partly to slow penetration of the adsorbed substances into the pores of the charcoal, partly to the slow development of irreversible changes The first cause does not affect the combination of antibodies with bacteria and red blood corpuscles, the second may account for the slow increase with time observed by Dreyer and Douglas

The relation of the amount of antibody combined to the concentration of antigen and of free antibody (Law of Mass Action and Adsorption isotherm) will be discussed at the end of the next chapter, since in many of the instances considered the degree of combination is affected by secondary reactions

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CHAPTER V

THE NATURE OF ANTIGEN-ANTIBODY REACTION: SECOND STAGE

In the usual immunity reactions the union of antibody and antigen is followed by a second stage It is probable that the more complex reactions, occurring in this second stage, are the result of changes similar to those which occur in the processes, precipitation and agglutination, which will be discussed here Before attempting an analysis of the nature of these changes it is as well to describe the effects of varying conditions on these two reactions

As has been pointed out in the first chapter the stability of suspensions depends on two factors—(1) the difference between the attraction of the particles for water and their mutual attraction, and (2) the surface potential of the particles Owing to the advantages of such materials for measurements of surface potentials, studies have been carried out mainly on particulate antigens such as bacteria Observations have been made on the surface potential, and on the stability of the suspension, and as a general rule the variations of the first factor have been deduced from these observations

A Effect of Salt in low and moderate Concentrations on the Stability of Suspensions of Particulate Antigens

The stability of bacterial suspensions is affected by several factors The classical work of Northrop and de Kruif (1921–2, a and b) with suspensions of *B typhosus* and Type D of the bacillus of rabbit septicaemia was done before the importance of differences between flagellated and non-flagellated and rough and smooth strains were appreciated It may be assumed that the strains used were smooth and flagellated The results of the interaction of various factors were peculiarly complicated The effect of increasing concentrations of various katıons on the surface potential and stability of suspensions of typhoid bacilli, at pH about 7, are shown in Fig XIX The effects on surface potential are similar to the effects on collodion particles (Fig XI) In low concentrations of salts the stability also is similarly affected, for the bacteria are agglutinated when the surface potential falls below a critical level of about 15 mvt In higher salt concentrations, however (e g 0 1 N NaCl), agglutination does not occur even when the surface potential falls well below this level It must be inferred that, when the salt concentration is raised, a change takes place on the surface of the bacteria, leading to a reduction of the mutual attraction of the bacteria or an increased attraction for water, a change from a hydrophobe to a hydrophil state

A reduction of the mutual attraction of the bacteria with rising salt concentration was demonstrated directly by Northrop and de Kruif (1921–2, a) by measuring the force required to separate two coverslips coated with the bacteria This effect of salt concentration on the

cohesion of the bacteria may be compared to the effect on the solubility of euglobulin, with this striking difference—the effect of solutions of salts of equivalent concentrations on the solubility of euglobulin increases with increasing valency, but this influence of valency is not found in the effect on the cohesion of bacteria Hence very low concentrations of polyvalent kations do not reduce the

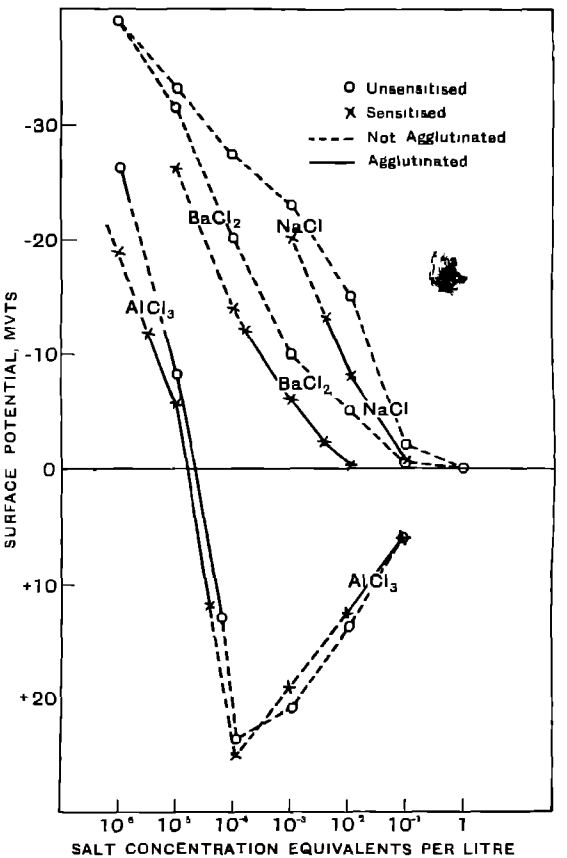


FIG XIX Effect of salt concentration on surface potential and agglutination of *B typhosus*, unsensitized and sensitized pH about 7–8 (Northrop and de Kruif, 1921–2, a and b)

mutual attraction of the bacteria, at the same time they reduce the surface potential below the 15 mvt required for stability, and therefore produce agglutination Monovalent kations, on the other hand, do not reduce the surface potential appreciably before the cohesion has been reduced also, and therefore do not cause agglutination in any concentration

Particulate antigens vary considerably in the two factors that affect the stability of suspensions The surface potential of Type D of the bacillus of rabbit septicaemia is relatively high, and it is therefore not readily agglutinated (Northrop and de Kruif, 1921–2, a). Rough strains of intestinal bacteria (e g *B typhosus, B flexneri, B. pullorum, B coli*) suspended in salt solutions are partially agglutinated when the surface potentials falls below 17mV and completely below 10mV. But the surface potentials of the smooth non-flagellated strains of the bacteria approach zero even in low salt concentrations , nevertheless, their suspensions are stable in any salt concentration (Joffe and Mudd, 1935) On the other hand, acid-fast bacilli, as might be expected owing to the lipoid nature of the surface, do not form hydrophil suspensions even in the higher salt concentrations Their suspensions are unstable when the surface potential of the bacteria is low

B Effect of Immune Serum on the Surface Potential and Stability of Suspensions of Particulate Antigens

In contrast with the variable and complicated behaviour of unsensitized bacteria, that of bacteria sensitized by agglutinating serum is fairly simple (Fig XIX) The effect of immune serum on the surface potential of particulate antigens is variable (Fig XX) At the pH (about 7–8) and salt concentration (0 9 per cent = 0 15 N NaCl), at which agglutination reactions are usually performed, the effect may be negligible , although at lower pH levels or salt

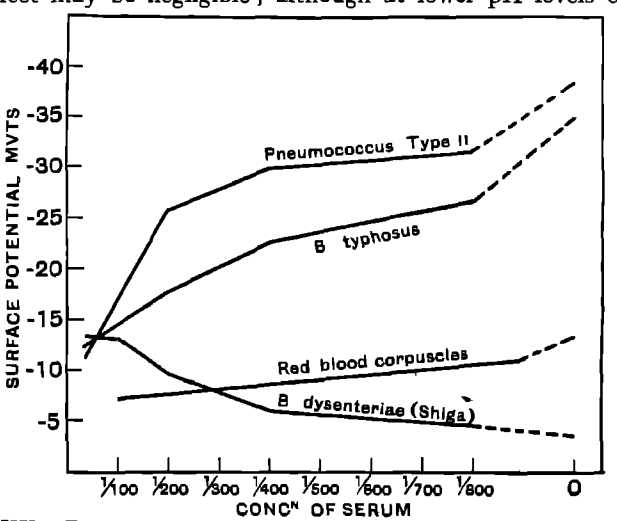


Fig XX Examples of the effect of immune serum on the surface potential of bacteria (Shibley, 1926) Note that this is in M/200 buffer solution In the 0 15 N NaCl solution usually employed the initial potential would be considerably lower (see Fig XIX) and effect of immune serum much less Also the effect of immune serum on the surface potential of red blood corpuscles in 0 15 N NaCl (Northrop and Freund, 1924)

concentrations the effect may be much more marked [1] Sensitization tends to bring the surface potential of all antigens to the approximately same level—that of serum globulin at the same pH and salt concentration This agrees with the facts already discussed that show that the antigens are coated with globulin from the antisera

The important effect of sensitization, however, is that the fully sensitized antigens behave like hydrophobe suspensions, even in moderately high salt concentrations According to the experiments of Northrop and de Kruif (1921–1922, b), with flagellated bacteria, the sensitized suspensions are dependent for their stability on their electric charges , the critical potential for all fully sensitized antigens appears to be about 13 mvt The effect of salt concentration on the surface potential of sensitized bacteria at pH 7–8 may be very similar to that on unsensitized bacteria (Fig XIX) The bacteria are not agglutinated unless a sufficient concentration of salt is present to reduce the surface potential below the critical level Polyvalent kations are more effective than monovalent, so that 0 0002 N $BaCl_2$, and 0 005 N NaCl are required to produce agglutination If, however, the antigens are not fully sensitized the change from hydrophil to hydrophobe is less complete, and the critical potential for agglutination is lower

When the pH is brought nearer the isoelectric point of serum globulin the surface potential of sensitized bacteria becomes progressively less, however low the salt concentration , the bacteria are therefore agglutinated in very low salt concentrations

TABLE XX

Effect of salt concentration on the flocculation of antigen-antibody complexes and heat-denatured globulin (Eagle, 1930, b)

		Concentration, gram equivalents per litre					
	Salt	*0 04*	*0 02*	*0 01*	*0 005*	*0 0025*	*0 00125*	*0 0006*
Sensitized *B coli*	NaCl	+++	+++	+++	+++	±		
	Na_2SO_4	+++	+++	+++	++			
	$BaCl_2$	++++	++++	+++	+++	+++	+++	++
Sensitized *B typhosus*	NaCl	++++	++++	++				
	Na_2SO_4	++++	+++	+				
	$BaCl_2$	++++	++++	++++	++++	+++	±	
Sensitized red blood corpuscles	NaCl	++++	++++	++++	+++	−		
	Na_2SO_4	++++	++++	++++	+	−		
	$BaCl_2$	++++	++++	++++	++++	++++	++++	++++
Protein anti protein precipitate	NaCl	++++	++++	++++	++			
	Na_2SO_4	++++	++++	++++				
	$BaCl_2$	++++	++++	++++	++++	++++	+++	−
Heat denatured serum globulin	NaCl	++++	++++	++				
	Na_2SO_4	++++	+++					
	$BaCl_2$	++++	++++	++++	++++	++++	+	

[1] Brown and Broom (1929) attach special importance to the reduction of charge that may occur on sensitization They consider that the absence of haemolysis on addition of complement in the absence of salt is due to the high negative charge of the particles , they misrepresent the work of Coulter (1920–1, b), which shows that at the pH used in immunity reactions the amount of haemolysin combined with the red corpuscles is much reduced in the absence of salt

As shown in Table XX salt concentration plays the same part in the flocculation of the antigen-antibody compound in precipitin reactions as in the production of agglutination in agglutinin reactions Flocculation also does not occur in salt-free mixtures of diphtheria toxin and antitoxin

C Effect of Low Salt Concentration

When the amount of agglutinin added to bacteria is very small, agglutination may occur only within a limited range of salt concentration (about 0 1 N according to Streng, 1909, and Northrop and de Kruif, 1921–1922, b) Duncan (1934 and 1937) has found that it is necessary to consider the three variables—concentration of the bacterial suspension, concentration of agglutinating serum, and concentration of salt Using smooth non-flagellated strains of *B typhosus* and *B enteritidis* (Gaertner), 8×10^8 organisms per c c , he found that, with the minimum serum concentration, complete agglutination occurred only in the presence of 0 0044 N sodium chloride As the serum concentration was raised agglutination also occurred in higher salt concentrations , but would no longer occur in concentrations as low as 0 0044 N At 1/5 dilution of a serum complete agglutination occurred from 0 018 N to 1 1 N salt Flagellated bacteria were agglutinated by minimum amounts of flagellar agglutinin only at a sodium chloride concentration of 0 0175 N , when the ratio of agglutinin to bacteria was raised, by varying either the serum concentration or the concentration of the bacteria, agglutination took place both at higher and lower salt concentrations (as found by Northrop and de Kruif) The minimum salt concentration at which agglutination would occur depended on the ratio of the concentrations of serum and bacteria, and not on the serum concentration alone

The differences in the behaviour of flagellated and non-flagellated bacteria may be, in part, due to the differences of surface potential Smooth non-flagellated bacteria have very low surface potentials , this potential is slightly raised when they are lightly sensitized It requires but low concentrations of salt to reduce this potential below the level needed to keep the bacteria apart , when they are more highly sensitized and therefore have a somewhat higher surface potential the salt concentration required is slightly higher It is necessary, however, to attribute a slight dispersive effect even to salt concentrations above the low level 0 007 M, to account for the failure of agglutination with higher salt concentrations when the sensitization is light With flagellated bacteria the salt concentration (0 0175 N) at which agglutination takes place with the minimum agglutinin is probably determined mainly by the degree of combination of agglutinin with bacteria, since maximum combination occurs at a near salt concentration (0 009 N) It is probable, however, that many factors such as the degree of aggregation of both the antigenic constituents of bacteria and of antibody are concerned in these effects of varied salt concentration

Duncan (1937) studied the salt concentration at which suspensions of bacteria were agglutinated most rapidly in the presence of antiserum He called these concentrations " salt optima ", on analogy with the optima found in the zone phenomenon, they are not the same as the salt concentrations in which the minimum ratio of serum to bacteria will produce agglutination These optimum salt concentrations are close to the concentrations at which maximum antibody is bound by the bacteria

D Effect of High Salt Concentration

Stronger concentrations of salt, e g 2 N, inhibit precipitin and agglutinin reactions (Streng, 1909, Friedberger and Goldschmidt, 1910, Landsteiner and Welecki, 1911) Agglutination is delayed in sodium chloride solutions over 0 5 N, precipitin reactions are delayed in concentrations over 0 2 N (Eagle, 1932), but still occur in 1 N solutions (Dean, 1931) Heidelberger, Kendall and Teorell (1936) found that the maximum antibody nitrogen precipitated by S S S III from antibody solutions, was reduced by high salt concentrations (Table XXI) No definite difference was found between the effects of mono- and divalent kations, and of mono- and divalent anions, the tetravalent ferrocyanide anion definitely counteracted the salt concentration effect The amount of precipitate formed by egg albumin and the corresponding rabbit antiserum was not affected by salt concentrations up to 1 75 M except in the region of antigen excess The flocculation of diphtheria toxin-antitoxin is delayed in high salt concentrations (Schmidt, 1930, a), the order of efficiency of anions in this inhibition (excluding those which affect pH) is the Hofmeister series

$$-ClO_4 > -SCN > -ClO_3 > -NO_3 > -Br > -IO_3 > -SO_4 > -Cl > -NO_2 > -F$$

Marrack and Smith (1930) found that diphtheria toxin-antitoxin floccules were dispersed by strong salt solutions, the order of efficiency was salicylate $> -I > -SCN > -Br > -NO_3$ Moderate variations in the concentration of salt (e g 0 06 to 0 15 N NaCl) caused no variation in the amount of precipitate in a precipitin reaction (Marrack and Smith, 1931, a) at optimum proportions

Table XXI

Effect of high salt concentration on amount of precipitate (Heidelberger Kendall and Teorell, 1936) in mg of nitrogen per 1 c c of serum

	Molar Salt Concentration				
	0 1	*0 15*	*0 51*	*0 93*	*1 79*
S S S III and horse antipneumococcal serum	1 78	1 66	1 54	1 28	1 22
S S S III and rabbit antipneumococcal serum		1 04		0 39	
Egg albumin and rabbit antiserum		1 32	1 32		1 32

TABLE XXII

Relation to pH agglutination of typhoid bacilli by immune and normal serum (Northrop and de Kruif, 1921–2, b)

Concentration of immune serum

	5/10 000	31/1,000,000	15 5/1 000,000	7 8/1,000,000	3 9/1,000,000	0
pH 8 5	C	C	++	—	—	—
,, 7 5	C	C	++	+	—	—
,, 5 5	C	C	++	+	—	—
,, 5 2	C	C	C	+	Tr	—
,, 5 0	C	C	C	++	+	—
,, 4 6	C	C	C	C	C	Tr
,, 3 9	C	C	C	C	C	C
,, 3 3	+	++	C	C	C	C
,, 2 7	+	+	+	+	+	+

Concentration of normal serum

	2/1,000	5/10 000	2 5/10,000	1 25/10,000	6/100 000	0
pH 5 0	Tr	—	—	—	—	—
,, 4 7	+	Tr	—	—	—	Tr
,, 4 4	+	Tr	+	++	++	Tr
,, 3 9	—	+	C	C	C	C
,, 3 3	—	+	+	++	++	C

E EFFECT OF HYDROGEN-ION CONCENTRATION

Michaelis and Davidsohn (1912) found that agglutinin and precipitin reactions were little affected by the reaction between pH 5 and 9 The effect of pH on agglutinin reactions is illustrated by Table XXII and Fig XXI With the higher antibody concentrations complete agglutination occurs over a wide range, with lower concentrations the effect of immune serum merges into that found with normal serum and with other proteins (see Chap I) and agglutination occurs only at low pH levels The measurements of Northrop and de Kruif (1921–2, b) show that with these low concentrations of antibody the surface potential lies below the critical level at the lower pH levels, but not at pH 6 or over (Fig XXI) This shift of the optimum pH towards lower levels, when the antibody concentration is reduced, is accounted for by the low isoelectric point of the bacteria The optimum pH for the agglutination of unsensitized red blood corpuscles is higher—pH 4 7—than that for the agglutination of unsensitized bacteria, the optimum pH for the agglutination of red corpuscles, sensitized with minimal amounts of agglutinin, is accordingly higher —about pH 5 3 (Coulter, 1920–1, a)

It is more difficult to explain the observation of Michaelis and Davidsohn (1912) with a precipitin reaction in which the antigen was sheep serum As the ratio of antibody to antigen was increased the pH range in which precipitation occurred shifted to the alkaline side As the isoelectric points of antigen and antibody must be about the same it would be expected that the pH optimum for precipitation would not be altered by altering the proportion of antigen to antibody.

It is possible that the optimum pH for the precipitation of the antibody used in these experiments was, like that of the pneumococcal antibodies, about pH 7

Sobotka and Friedlander (1928) found that, with reduction of pH, the minimum concentration of specific pneumococcal polysaccharide required to produce a precipitate with higher concentrations of antiserum (e g 1/20), was reduced This may be ascribed to the lower charge at the lower pH levels They also found, using citrate buffers, that with low concentrations of antibody and high concentrations of antigen precipitation did not occur at the lower pH levels (e g pH 5) This may have been due to a special effect of the citrate ion

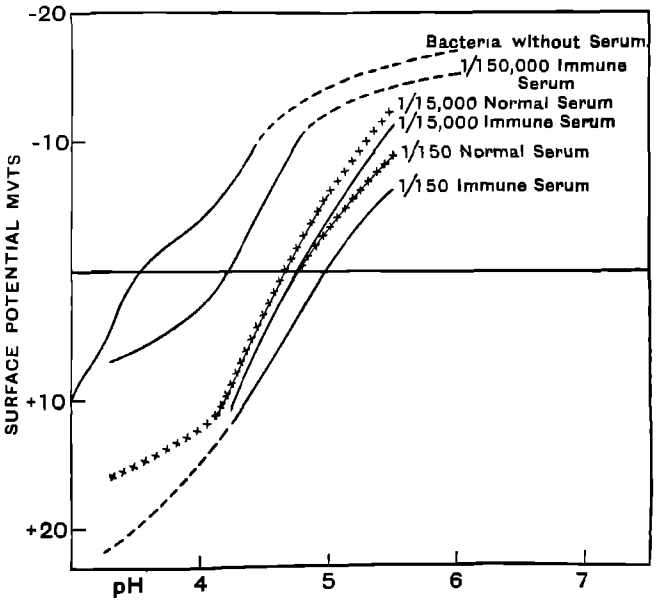


FIG XXI Effect of immune and normal sera on the surface potential of typhoid bacilli at various pH levels Unbroken lines = agglutination (Northrop and de Kruif, 1921–2, a and b)

Mason (1922) found that precipitation reactions occurred between pH 4 5 and 9 5, outside this range immune precipitates dissolved Watson and Longstaff (1926) found that flocculation of purified diphtheria toxoid and antitoxin is greatly delayed at pH 9 and 10. According to Schmidt (1930, c) the rapidity of flocculation of diphtheria toxin and antitoxin is almost constant from pH 5 55 to pH 8 At pH 4 5, 4 95, and 9 5 flocculation is very slow, and it does not occur at pH 10 Diphtheria toxin-antitoxin floccules suspended in a weak NaCl solution (about 0 01 N) begin to disperse at about pH 9 and pH 4, and give a clear solution at pH 3 Brown (1935)

found the precipitate formation by S S S I and antiserum is delayed below pH 6 0

Within moderate pH ranges (e g 6 6–8 0) the amount of precipitate is constant, this was found by Marrack and Smith (1930, 1931, a) with the antigens diphtheria toxin and serum globulin and the corresponding antibodies at optimum proportions, also by Heidelberger and Kendall (1935, c and d) with egg albumin and an azo-protein as antigens In the case of egg albumin there was no reduction of the amount of precipitates obtained at pH 6 36 even in the inhibition zone, with the azo-protein the gradient of the inhibition zone was possibly slightly steeper at pH 6 7 than at 7 9

The titration curve of diphtheria toxin-antitoxin floccules resembles that of serum pseudoglobulin (Marrack and Smith, 1930) Hirsch (1922) described changes in the pH in immunity reactions such as would result from the union of a weak acid with a strong base In these experiments large amounts of buffer substances were present which would conceal any change that might occur, and apparently no precautions were taken to prevent changes of reaction from other causes Smith and Marrack (1930, a), avoiding these objections as far as possible, found no change of pH

F Effect of Temperature

The rate of precipitation or agglutination usually rises rapidly from 0° to 30° C Above 30° the rate still increases, but less rapidly, and the reaction may sometimes be slower at 56° C than at 37° C (Eagle, 1932) Above 56° C the antibody begins to be affected In some cases the inhibiting effect of higher temperatures is more striking Ottensooser (1923) found that the precipitate formed by egg albumin and antibody dissolved at 55° C, the precipitate did not form again on cooling, so that it appears that the antibody or antigen may have been damaged even by this temperature However, Heidelberger and Kendall (1929, c) found that the precipitate formed by Type III pneumococcal polysaccharide and antibody, in certain proportions, dissolved on warming from 0° C to 37° C or even to room temperature, and reappeared on cooling Later (1935, b) they showed that, over the whole range of antibody/antigen ratios, the amount of protein precipitated by S S S III from horse antiserum at 37° C was considerably less than that precipitated at 0° C In contrast with this the amount and composition of precipitates formed by azo-protein and by egg albumin and the corresponding rabbit antisera are little affected by change of temperature from 37° to 0° C (Heidelberger and Kendall, 1935, c and d) Duncan (1932, a) found that resolution of the precipitate formed by yeast gum and antiserum occurred at 50° C, followed by slow re-precipitation on cooling Dean (1912) obtained similar results with an extract of *B typhosus* and antityphoid serum He connected this with the binding of complement, but complement cannot have been involved in the experiment of Heidelberger and Kendall (1929, c) in which purified, matured antibody was used

Bayne-Jones (1925) described a surprisingly high heat formation in the neutralization of toxin by antitoxin, Smith and Marrack (1930, a) were unable to confirm this

G Effect of Changes in Antisera

Reduction or disappearance of agglutination or precipitation may result from changes in antibody by which the power of combining with antigen is unaffected Diphtheria antitoxin precipitated with sodium sulphate neutralizes toxin, but does not flocculate Ramon (1922, c) prepared a pseudoglobulin fraction from diphtheria antitoxic serum which would not flocculate with toxin Jones (1928, a) found that hog-cholera agglutinin heated at 75° C for 30′ would not agglutinate bacteria, but apparently combined with them as it rendered them inagglutinable by unheated agglutinin The effects of various chemical treatments of the proteins of antisera on their antibody properties has been mentioned in Chapter II, and will be discussed further in the section dealing with the unitarian hypothesis The effects of extracting alcohol- and ether-soluble substances will be discussed later (Section L)

H Effect of non-specific Serum Proteins

In view of the protective and precipitating effects of proteins (Chap I) it is to be expected that non-specific proteins may affect agglutination and precipitin reactions and that the effect may be in either direction Dean (1912) found that euglobulin, prepared from normal guinea-pig serum by dilution and the passage of CO_2, increased the degree of precipitation or agglutination when antigen was in excess The active substance was not destroyed by heating at 56° C. for half an hour Eagle (1930, a) showed that the fraction of fresh normal serum which accelerated the agglutination of sensitized red blood corpuscles was that precipitated by dilution and the passage of CO_2 The acceleration ran parallel to the mid-piece complementary activity of the serum, and the effect of heated serum was due to residual mid-piece, not destroyed by the heating Marrack and Smith (1931, b) found that the addition of normal rabbit serum increased the amount of precipitate formed by azo-protein and precipitin only when the antigen was in excess, but that the precipitate formed by iodo-protein and the corresponding precipitin was increased even at optimum proportions. The ratio of antigen to total protein in the precipitate was not affected in either case The normal serum therefore seems to have caused more complete precipitation of the antigen-antibody compound Sobotka and Friedlander (1928) found that the addition of normal horse serum considerably increased the dilution of antibody which would give a precipitate on the addition of specific polysaccharide Such effects, however, are by no means invariable Addition of non-specific proteins may have no effect Dean(1912) did not demonstrate any difference when antigen was not in excess Marrack and Smith (1931, a) found that guinea-pig euglobulin, fresh guinea-pig serum, or human serum did not affect

the amount of precipitate in a precipitin reaction, even when antigen was in considerable excess, they also found the addition of non-specific proteins had no effect on the amount of precipitate obtained from balanced mixtures of diphtheria toxin and antitoxin An opposite effect, reduction of the sensitivity of the reaction, has been noted by Sobotka and Friedlander (1928) when antibody was in excess White (1931) found that the somatic agglutination of the salmonella group was inhibited by normal rat- or rabbit-serum, and that the addition of fresh serum, diluted 1/5 to 1/20, to agglutinated bacteria caused their more or less complete dispersal On the other hand, according to Duncan (1934), normal serum, in a final concentration as high as 1/16, slightly retards somatic agglutination of Flexner dysentery bacilli (smooth and non-flagellated) when the amount of antibody corresponds to the β ratio (see Section M, 2) When either antibody or antigen is in excess normal serum accelerates agglutination and extends the range of antibody dilution that will produce complete agglutination

Maltaner and Johnston (1921) consider that the effect of fresh serum on the rate and completeness of agglutination is due to residual fibrinogen in the serum It is probable that the action is a complex one, in some cases residual fibrinogen may be the most active constituent in the serum, in other cases the antagonistic actions of serum albumin and globulin may be involved, as in the sedimentation of red blood corpuscles (see Chap I)

I Effect of non-specific Organic Substances

Various organic substances have an inhibiting effect on the precipitin and agglutinin reactions The explanation given of the protective action of these substances is that they form a layer on particles, the polar groups of the layer being turned towards the water Landsteiner and Welecki (1911) found that cane sugar in concentrations over normal (34 per cent) reduced the amount of precipitate in precipitin reactions Glucose in normal (19 8 per cent) or twice normal concentration reduced the amount of precipitate in precipitin reactions (Landsteiner and Welecki, 1911) or inhibited the reaction altogether (Downs and Goodner, 1926), concentrations of 50 to 100 per cent inhibited agglutination reactions (Kritschewski and Awrech, 1929) Bayer 205, which in 0 1 per cent concentration prevents the coagulation of albumin by tannin, by mercuric chloride and by boiling (Jirovec and Kocián, 1930), inhibits bacterial agglutination in 2 per cent concentration, and precipitin reactions in 4 to 6 per cent concentration (Kritschewski and Awrech, 1929) Eisenberg and Volk (1902) found that saturated solutions of urea considerably increased the amount of agglutinin to produce agglutination, and reduced the combination between agglutinin and bacteria But Landsteiner and Welecki (1911) found that 12 per cent solutions (2 N) had no effect on precipitin reactions

Surprisingly little work has been done on the effects of surface-active substances on immunity reactions. Takenomata (1924)

found that saponin checked precipitin, but not agglutinin, reactions Presumably the extent of the inhibition depends on (1) the relative strength of the non-specific attraction of the antigen surface for saponin and the specific attraction of this surface for the antibody, and (2) the attraction for water of the molecules of saponin which have been adsorbed on areas of antigen-antibody complex other than the specific combining sites, which prevents aggregation

J Nature of Antigen-Antibody Precipitate

1 *Composition*

The principal constituent of antigen-antibody precipitates is protein derived from the antiserum Antigen is also present and suitable antigens can be recognized and recovered The ratio of antibody to antigen in the precipitate and its relation to the proportions in which the reagents are mixed will be discussed later Numerous analyses (Table XV, p 62) suggest that these precipitates cannot contain large amounts of material other than protein Douglas and Dudley (quoted by Hartley, 1925, b) found 25 per cent of the precipitate from a precipitin reaction soluble in fat-solvents Marrack and Smith (1930, 1931, a) found less than 1 per cent of such material in diphtheria toxin-antitoxin floccules, and in the precipitate formed by horse pseudo-globulin and its precipitin they could not detect the presence of cholesterol Breinl and Haurowitz (1930) found, in the precipitate given by haemoglobin and antiserum, from 2 to 8 per cent of lipin, in which no galactose, phosphorus, or cholesterol was detectable The high percentage of lipin found in diphtheria toxin-antitoxin floccules by Flossner and Kutscher (1924) was probably due to the greater part of the protein being removed by washing with distilled water The ammonium phosphate which they also found must have been an accidental contamination from the toxin broth

The amount and nature of the lipin in specific precipitates formed by S S S I has been more fully studied by Horsfall and Goodner (1936, a and b) When the concentration of antiserum in the reacting mixture was kept constant and the antigen varied the amount of lipin in the precipitate was approximately constant The lipin therefore formed a high proportion (up to 58 per cent) of the precipitate when this was small, and a low proportion (down to 3 8 per cent) when the precipitate was large The lipin consisted of free cholesterol, cholesterol esters and phosphatides The precipitates from rabbit antisera contained very little lipin amino N, which indicates that the phosphatide was not cephalin Half the lipin N of precipitates from horse serum was amino N

2 *Solubility*

Wu, Sah, and Li (1929), working with iodo-proteins as antigens, found the amount of precipitate little affected by washing with 0 9 per cent NaCl solution This was also found by Heidelberger and

Kendall (1933) using S S S III as antigen, and by Taylor, Adair and Adair (1934) with egg albumin as antigen Heidelberger and Kendall also obtained the same values for the precipitate nitrogen whether this was estimated directly, after washing, or indirectly by subtracting the nitrogen in the supernatant fluid from that original present in the reacting mixture However, Felton (1932) found that, when the precipitates formed by S S S I and II and horse antipneumococcal sera were washed with 0 85 per cent solution of NaCl containing 1/20,000 S S S, the washings contained 0 04 and 0 06 mg of N (0 25 and 0 38 mg of protein) in 1 c c In this instance a small amount of antibody may have dissolved out in combination with excess of antigen in the washing fluid

The effect of diluting the reacting mixture of antigen and antibody has been studied by Marrack and Smith (1931, a) using horse pseudoglobulin as antigen, and by Heidelberger and Kendall (1935, a and d) with S S S III and egg albumin as antigens With the protein antigens the reduction of the amount of precipitate when the volume was increased indicated a solubility of about 0 003 to 0 005 mg N in 1 c c But with S S S III dilution caused little or no reduction in the amount of precipitate Solution of antigen-antibody compounds may be due to the solubility of these compounds as such, or to their dissociation into its constituents We have evidence of dissociation in the effects of dilution on neutral mixtures of diphtheria toxin and weakly avid antitoxin, which is the basis of the dilution ratio of Glenny and Barr (1932) Antigen-antibody precipitates appear to become less soluble on keeping, this applies particularly to diphtheria toxin-antitoxin floccules[1] A further relatively irreversible change appears to take place

The question whether antigen and antibody can exist together in solution is closely connected with the solubility of the precipitate. As the ratio of antigen to antibody is increased considerably above optimum proportions, the amount of globulin precipitated from a given amount of antiserum and the proportion of the antigen added that is precipitated both diminish (Marrack and Smith, 1931, b) It is obvious that, under these conditions, a certain amount of both antibody and antigen has not been precipitated Dean (1931) reports that at optimum proportions both antigen and antibody disappear almost completely from the supernatant fluid in a precipitin reaction Culbertson (1932), using egg-albumin as antigen, could detect neither antigen nor antibody in the supernatant fluid at the neutral point This was also found by Taylor, Adair and Adair (1934) and Heidelberger and Kendall (1935, d) On the other hand, Heidelberger and Kendall (1929, c, 1935, b) found that, with S S S III as antigen, both antigen and antibody might, in some cases, be present in the supernatant fluid Taylor, Adair and Adair found both antigen and antibody in the supernatant fluid, at optimum proportions of serum albumin and antiserum The simultaneous

[1] Also Ottensooser (1923) found that precipitates kept 24 hours would not dissolve on heating to 55° C, although fresh precipitates would dissolve

presence of unprecipitated antigen and antibody was probably due to non-uniformity of both At a certain ratio, modified antigen and weak antibody are left in the supernatant Neither of these will form a precipitate with the other, but the modified antigen will form a precipitate with more active antibody, and the weak antibody with unmodified antigen Preparations of Type III polysaccharide are not uniform (Heidelberger, Kendall and Scherp, 1936), nor are the antibody molecules of antipneumococcal sera (Heidelberger and Kendall, 1935, b) There is evidence that preparations of serum albumin, such as were used by Taylor, Adair and Adair, are not uniform When coloured antigens, such as azo-proteins, are used, incomplete precipitation, even at optimum proportions, may be inferred from the colour of the supernatant fluid This colour is not due to non-protein impurities, as it is practically completely removed by excess of antibody Heidelberger and Kendall (1935, c) have shown in the case of one azo-protein antigen that a proportion, depending on the antiserum used, is not precipitated by antiserum It is probable that discrepancies between the results found by various workers are due to variations in the firmness of combination of antigen and antibody, the degree of uniformity of antigen and antibody, and of the extent to which irreversible changes of the antigen-antibody complex occur

3 *Reversibility of the Reaction*

Unless it is assumed that antigen and antibody form irreversible compounds it is difficult to explain the observation that the composition of precipitate in a precipitation reaction depends not on the final concentration of antigen and antibody in the supernatant fluid, but on the total amounts of these present (Heidelberger and Kendall, 1935, b) Other evidence suggests that the reversibility of these reactions varies considerably If a reaction is reversible the same result should be obtained from the mixture of given amounts of the various reagents, in whatever order they are mixed The familiar example of irreversibility is the Danysz reaction given by diphtheria and tetanus toxin and the corresponding antitoxins [1] A more striking example is that described by Burnet (1931, a) He found that the compound of staphylococcal toxin or toxoid with antitoxin became irreversible within five minutes Ramon (1930) found that a lethal dose of diphtheria toxin was displaced from combination with a slowly flocculating antibody by one-fifth the amount of toxoid that was required to displace it from a rapidly flocculating antibody This is presumably due to differences in the

[1] The phenomenon studied by Heidelberger and Kendall (1935, b) is not strictly comparable They found that, for example, a given amount of S S S III precipitated more antibody from a given volume of an antibody solution if it was added in a series of small parts (each added after the precipitate formed by the previous part had been removed) than if it was added all together This phenomenon depends solely on the fact that the ratio of antibody to antigen in the precipitate is higher when antibody is in excess, and not on irreversibility.

degree of reversibility of the combination of toxin and antitoxin, or of secondary changes In the case of diphtheria toxin and excess of antitoxin, however, we are not dealing with true irreversibility but slow reversibility Healey and Pinfield (1935) have shown that the amount of antitoxin finally left free is the same, depending solely on the final amounts of toxin and antitoxin present in the mixture, whether this is made by mixing toxin and antitoxin directly in the final proportions, or by mixing a neutral mixture of toxin and antitoxin, after flocculation, with excess of antitoxin and allowing to stand

The Danysz phenomenon can also be demonstrated by the Ramon flocculation method If 1 c c of toxin is mixed with a series of doses of antitoxin and later another c c of toxin is added, flocculation occurs first in a tube containing more antitoxin than would be contained in the tube that would flocculate first if the antitoxin were added to the whole 2 c c of toxin at once

Although the combination of haemolysin with red blood corpuscles was found by Cromwell (1923) to be completely reversible, a phenomenon similar to that of Danysz was observed by Philosophow (1909) He added an excess of haemolysin to a given amount of red blood corpuscles, after standing, these were centrifuged off and added to a second equal amount of red corpuscles The second batch of corpuscles was haemolysed to some extent This haemolysis must have been produced by haemolysin rendered available by reversal of its combination with the first batch If six or more haemolytic doses were added to the first lot of corpuscles haemolysis of the second batch was complete, so that one haemolytic dose must have been given up by the first batch Only a trace of haemolysin was left in the supernatant fluid from the first batch when six haemolytic doses were added If less than six doses were added to the first batch, haemolysis of the second batch was not complete So that whereas the two batches of corpuscles, if added to haemolysin in one lot, would only require two doses of haemolysin to produce complete haemolysis, they required six then added in two lots The dissociation of haemolysin took place more rapidly at 40° C than at 20° C or 0° C It was not affected by the length of time that the first batch of corpuscles was left in contact with haemolysin If, instead of 0 85 per cent NaCl, isotonic solutions of KCl or $CaCl_2$ were used for dilution, the dissociation of the haemolysin from the first batch of red corpuscles was complete and only two doses were required to haemolyse the two batches of red corpuscles added in sequence

No precipitate is formed in precipitin reactions when antibody is added to a considerable excess of antigen If the process was fully reversible an antigen-antibody precipitate should dissolve in a strong solution of antigen Breinl and Haurowitz (1930), however, found that the haemoglobin-antihaemoglobin precipitate would not dissolve in haemoglobin solution Sobotka and Friedlander (1928) found that the precipitate formed by Types I and II pneumococcal antibodies and antisera, after being kept 24 hours, would not dissolve in 0 1 per cent

solution of homologous polysaccharide Heidelberger and Kendall (1929, c), however, found that Type III polysaccharide-antibody precipitate, also kept 24 hours, would dissolve in 1 per cent polysaccharide solution Healey and Pinfield (1935) found that diphtheria-toxin-antitoxin floccules would dissolve in excess either of antibody or antigen The precipitate formed by haemocyanin and antibody dissolves on standing overnight in excess antigen (Hooker and Boyd, 1936) Precipitates formed by azo-proteins and antibodies dissolve in excess of simple substances containing the determinant group (Landsteiner, 1921)

K Nature of the Secondary Reaction between Antigen and Antibody

Since the antibody globulin coats sensitized particulate antigens and forms the greater part of antigen-antibody precipitates, the new properties of the antigen-antibody complex may be ascribed to changes in the antibody globulin It appears that these properties are very similar to those of proteins denatured, for example, by heat Heat-denatured proteins are flocculated when their surface potential has fallen below a critical level, and the flocculation of heat-denatured serum proteins is inhibited similarly in the presence of higher salt concentrations (Chick and Martin, 1912) The efficiency of different salts, and the concentrations required for peptizing proteins which have been denatured by heat and flocculated (Willheim, 1929), is similar to that found for peptizing diphtheria-toxin-antitoxin floccules Heat-denatured serum proteins are peptized by acid and alkali in low salt concentrations, as are antibody-antigen precipitates The solubility of proteins precipitated by alcohol undergoes a gradual diminution on standing, similar to that found with some antigen-antibody precipitates (Merrill and Fleischer, 1932)

Eagle (1930, b) has emphasized this similarity to the denaturation of protein by heat and in other ways He has suggested that the loss of affinity for water is due to the formation of a layer of antibody globulin on the antigen, with the polar groups of the antibody turned towards polar groups of the antigen, and the non-polar groups of the antibody turned towards the water [1] Such a film would resemble a condensed protein film (see Chap I) except that the polar groups would be turned away from the water instead of towards it Such a change need not involve a drastic rearrangement of the protein molecule, if the formation of a layer involves only the separation of laminae, or even the unfolding of a lamina that was folded up into a globular form, as suggested by Wrinch (1937) However, two considerations make it seem unlikely that the antibody is spread in a thin film on the surface of the antigen In the first place, the whole surface of one molecule of globulin could be covered by another in a layer about 10 Å thick In actual fact we find that when serum

[1] In the case of egg albumin the formation of surface films leads to denaturation This, however, does not appear to be the case with plasma proteins (Wu and Ling, 1927).

globulin is used as antigen and mixed with antibody in the proportions which flocculate most rapidly, one molecule of globulin antigen combines with about four antibody molecules (Marrack and Smith, 1931, a) If all these are in contact with the antigen they cannot be spread out Similarly 0 5 mg of Type III pneumococcal polysaccharide combines with 27 mg of antibody globulin (Heidelberger and Kendall, 1929, c) Even if the polysaccharide was spread as a thin sheet, thus presenting the maximum surface and antibody was deposited on both sides, the protein layer would be over 27 Å thick Boyd and Hooker (1934, 1936) calculate that, at optimum proportions, the antigen is coated by a layer of antibody equivalent to a series of spheres of diameter approximately 44 Å

Also, if, as suggested by Eagle, the polar groups of the antibody protein are turned towards the antigen and non-polar parts are turned towards the water, the electro-kinetic properties of the antigen-antibody complex should resemble those of particles of some non-polar substance such as paraffin oil, and not, as actually found, those of protein

The differences of molecular arrangement of the antibody when adsorbed on the surface of water and when adsorbed to antigen would lie in this , the water strongly attracts all polar groups, and the antibody molecule is spread out to bring the maximum number of these in contact with the water surface On the other hand, the antigen strongly attracts only certain patches on the surface of the antibody , these patches are brought into contact with the determinant sites of the antigen, but no great distortion of the antibody molecule is possible , only such as will not seriously affect the surface character

It has been pointed out that completely denatured proteins have reverted to the fibrous form, a change detectable by X-ray analysis Two specific precipitates from anti-pneumococcal sera Types I and II examined by Astbury (private communication) without drying were not in the fibrous form , there was no evidence that the protein has changed from the globular form A complete denaturation comparable to that producible by heat can therefore be ruled out

When we consider the close packing of antibody molecules round the antigen, it does not appear necessary to invoke any special process of denaturation to account for the formation of aggregates When the antibody molecules are attached to the antigen (Fig XXIIA), the polar groups, on which the solubility of the antibody globulin normally depends, are brought into apposition with each other, and attract each other instead of water molecules[1] (see Chap I, p 20) The effect of such close packing on the accessibility of polar groups to water is well illustrated in the case of the fibrous proteins (Jordan Lloyd, 1933) Only the polar groups on the free surface are left available for binding water If these are insufficient to keep the complex in solution, the complexes will aggregate if the surface

[1] The titration curve of the antibody globulin need not be altered by this union That of wool keratin is not affected by the binding together of the chains.

potential is below a critical level If the antibody molecule has more than one adsorbing site it is possible that the complexes may be bound together in a coarse lattice such as is shown diagrammatically in two dimensions in Fig XXIIB This view differs from the hypothesis that the antibody globulin is denatured in that the aggregation of the particles of antibody combined with antigen is ascribed not only to a loss of attraction for water, but also to a specific attraction between the particles This mutual attraction is due to the link provided by further antigen molecules[1] Differences in the structures thus formed would account for the macroscopic differences between the precipitates formed in different reactions Polysaccharide antigens, for example, which probably

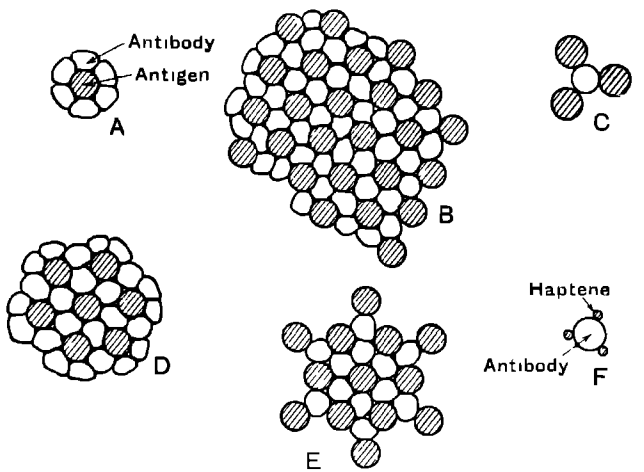


FIG XXII Diagrams of possible arrangements of antigen and antibody molecules in the complex (A) simple unit, (B) complex structure at optimum proportions, (C) antigen excess, (D) antibody excess, (E) antigen excess, (F) antibody and haptene

form long chain molecules (Heidelberger and Kendall, 1932, a), can bind together a large structure, forming disk-like coherent precipitates, unlike the granular precipitates formed by most protein antigens

Certain antigens are undoubtedly multivalent Type III polysaccharide is a polymer of an aldobionic acid, and must contain the combining groups repeated many times The synthetic azo-proteins

[1] Reduction of the charges on the particles by moderate salt concentrations will assist their aggregation whether this be due to loss of attraction for water or a specific mutual attraction For example, on the alkaline side of the isoelectric point, the combination of nucleic acid (negatively charged) and egg albumin (also negatively charged) only occurs in the presence of salt, on the other hand, salt reduces the combination between nucleic acid and albumin (positively charged) on the acid side of the isoelectric point (Przylecki and Grinberg, 1933)

do not form precipitates with antibody unless they contain several determinant groups to the molecule For example, antigens formed from *p*-amino-benzene-arsinic acid must contain at least one per cent of arsenic (Hooker and Boyd, 1932) It has already been suggested in Chapter III that natural protein antigens have several determinant groups per molecule In the same way it may be expected that the antibody sites are repeated in the antibody protein molecule

If both antigens and antibodies have several combining groups per molecule it seems that this specific combination must play a part in the formation of aggregates This may, however, not be the only factor The environment of antibody molecules when attached to antigen is entirely different from that of molecules in solution In solution the antibody protein molecules take up the position that will expose the maximum number of polar groups to the freely moving polar water molecules and to ions in solution But relative movements of the polar groups of the antigens are limited, even if the antigen molecules are susceptible to a considerable degree of deformation Apart from the attraction between non-specific polar groups, there is the special attraction between binding sites and determinant groups, whose relative movement is again limited The antibody molecules must therefore be subject to some degree of deformation This deformation is comparable to the spreading out of proteins on water, being also produced by the attraction of polar groups whose range of movement is limited, though the resultant distortion may be entirely different But because of the mutual attraction between polar groups of antigen and antibody, to the exclusion of the polar water molecules, the solubility of the antibody will be reduced

It is, therefore, suggested that aggregates of antibody and antigen are built up by union of specific bonds, and that the solubility of the aggregates is reduced by distortion of the antibody molecules, with blocking of polar groups

Four attempts have been made to test the theory of aggregation by specific bonds If two antigens, g_1 and g_2, are mixed with corresponding antibodies A_1 and A_2, then on the specific bond hypothesis $g_1 A_1$, $g_1 A_1$, $g_1 A_1$, and $g_2 A_2$, $g_2 A_2$, $g_2 A_2$ should aggregate separately On the theory that aggregation is due to the non-specific agglutination of hydrophobe particles of low surface potential, mixed aggregates $g_1 A_1$, $g_2 A_2$, should form Topley, Wilson and Duncan (1935) found that various intestinal bacteria and pneumococci agglutinate separately according to the specific bond theory However, Abramson (1935) obtained mixed aggregates of sheeps' red blood corpuscles and Freidlander s bacilli, and Hooker & Boyd (private communication) mixed clumps of human and chicken red blood corpuscles , the chicken corpuscles were distinguished by their nuclei

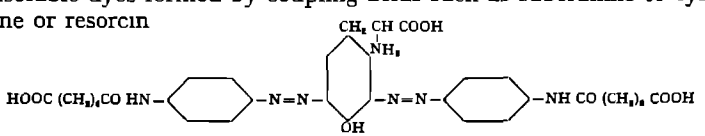
Also Hooker (1936) found that the rate of particulation in the region of excess antibody was proportional to the total concentration

of antigen, not only in a single antigen-antibody system, but also when two unrelated systems were involved

In three of the four tests, therefore, the aggregation is non-specific It may be suggested that in the experiments of Topley, Wilson and Duncan the agglutination of the two species of bacteria were not exactly simultaneous However, mixed agglutination of such relatively large particles does not exclude the aggregation of antigen and antibody molecules by specific bonds on the surface of the particles, with, as has been suggested above, blocking of polar groups and subsequent aggregation of the large particles non-specifically

It was suggested in the first chapter that certain globulin molecules were less soluble or more readily changed into a less soluble form, and that this difference may possibly be due to associated lipins It is possible that antibody globulin molecules which have this character, and are therefore more easily precipitated in the various fractionation methods, aggregate and form specific precipitates more readily, as suggested by Schmidt (1933) It is significant that the antibody to Pneumococcus Type III in horse antiserum is insoluble in distilled water and gives precipitates with fractions of the specific polysaccharide formed by hydrolysis , whereas rabbit antiserum is not precipitated by dilution with distilled water, and will not give precipitates with the fractions of the polysaccharides (Heidelberger and Kendall, 1933) Also that the antitoxin fractions precipitated by the lower concentrations of ammonium sulphate form less reversible combinations with diphtheria toxin than do the fractions precipitated by higher concentrations (e g Glenny and Barr, 1932).

If only two determinant groups are attached to one molecule an aggregate of unlimited size (AGAGAG———) can be built up However, the union between individual combining sites and determinant groups is weak, as noted in Chapter IV When adjacent molecules are held together by a few such weak bonds a large aggregate is liable to be shaken to pieces by thermal agitation Apart from this, the larger the number of molecules to which the determinant groups are attached the greater their relative mobility and the less the deformation of the antibody molecules With simple haptene molecules which have only one determinant group neither building up of aggregates nor deformation of antibody molecules can, in general, occur (Fig XXII F) Landsteiner and van der Scheer (1932), however, have found that certain relatively insoluble dyes formed by coupling acids such as suberanilic to tyrosine or resorcin



form precipitates with the antisera produced by immunizing with these compounds coupled to proteins It is probable that these dye molecules are strongly aggregated themselves when in solution, and therefore provide a basis for the close packing of antibody molecules

When antigen is in excess complexes such as C or E in Fig. XXII will appear[1] Complexes such as C will remain soluble, as the polar groups of the antigen molecules are not brought together, and will therefore not aggregate, complexes such as E surrounded by antigen groups will be soluble and cannot be bound together by specific bonds, as none of the aggregates have any free antibody molecules on the surface Actually ultracentrifuge methods show that aggregates containing a few molecules only are formed when antigen is in great excess (Heidelberger and Pedersen, 1937) When antibody is in excess complexes such as D will be formed which likewise cannot be bound together by specific bonds Consequently aggregation will be hindered when either antibody or antigen is in excess, giving rise to the so-called zone phenomenon Before further theoretical consideration of this inhibition by excess, it is as well to discuss the characteristics of the zone phenomenon as actually observed

L The Role of Lipins in Immunity Reactions

The amount of lipin in the serum may, in some cases, be increased after immunization Danysz-Michel and Laskowncki (1924) found cholesterol increased in the serum of a rabbit immunized with *B paratyphosus B* Ionesco-Mihaiesti and Damboviceanu (1930) also found that cholesterol rose in the serum of a horse immunized with the same bacillus, reaching a maximum at the same time as the agglutination titre, but falling in two months although the titre remained high Felton and Kauffmann (1933) found a series of antisera to pneumococcus Types I and II to contain an average of 1 44 per cent of constituents soluble in alcohol and chloroform, as compared with the normal 1 04 per cent However, Marie (1923) found that various immune horse-sera contained less cholesterol than normal, and Koldaev (1921) that the sera of horses immunized against tetanus, diphtheria, and typhoid bacilli contained the same amount of cholesterol as normal sera Danysz-Michel and Laskowncki also found that the injection of non-antigenic substances, such as Lugol's solution, caused a rise of serum cholesterol

Felton and Kauffmann (1933), finding that a ten times concentrated solution of antibody contained no higher concentration of lipin than normal serum, concluded that the actual increase of lipin in a serum cannot be essential to its antibody properties However, Chow and Goebel (1935) found 2 5 per cent of lipin in specially purified antibody globulin of which 90 per cent was precipitable

[1] Taylor (1931) has suggested that antigen and antibody in optimum proportions are united chemically and that any further antigen is attached to the compound so formed by adsorption Such " adsorption " must be regarded as specific, otherwise why should the homologous antigen only be adsorbed (see Heidelberger and Landsteiner, 1923, and Marrack and Smith, 1931, b) It seems superfluous to invoke two specific mechanisms by which an antibody can bind its antigen Comparison may be made to the surplus I^- ions " adsorbed " by an Ag I precipitate, when I^- is in excess In this case the " adsorbed " ions are attached to the space lattice by exactly the same forces as bind the Ag^+ and I^- ions in the bulk of the precipitate

The essential change in antisera need not be a general increase of lipins but an increase of some special constituent, a change in the association of some lipoidal constituent with serum proteins, or of the ratios in which various constituents are present Also the lipin normally present may be essential to some reactions

The effect of removing alcohol- and ether-soluble substances from antisera, without apparent denaturation of the proteins, was thoroughly studied by Hartley (1925, b) Horsfall and Goodner (1935) investigated the effect of lipin extraction on the reactions of Type I antipneumococcal serum In this case there is no question of the presence of lipin in the pure antigen S S S I The results of the two investigations are most conveniently shown in Table XXIII

TABLE XXIII

Effect of extraction of Lipin on immunity reactions

Antiserum	*Method of Extraction*	*Nature of Reaction*	*Antibody Extracted*	*Antigen Extracted*	*Effect*
Rabbit anti-horse serum	Alcohol and ether in cold	Precipitation	No	Yes	Amount of precipitate and range of precipitation reduced
,,	,,	,,	Yes	No	,,
,,	,,	,,	Yes	Yes	No precipitate
Horse diphtheria antitoxin	,,	Flocculation	Yes	No	No flocculation
,,	,,	Neutralization of toxin	,,	,,	Very slightly reduced
Horse (?) against *B typhosus*	,,	Agglutination	,,	,,	Unaffected
Horse against sheep's red blood corpuscles	,	Haemolysis	,	,,	,,
Human syphilitic	,,	Complement fixation	,,	,,	No fixation
Horse , Type I anti-pneumococcal	,	Precipitation	,,	,,	No precipitate
		Agglutination			No agglutination
		Protection			Unaffected
Rabbit , Type I anti-pneumococcal	,,	Precipitation	,	,,	Very slightly reduced
		Agglutination			
,, ,,	Alcohol, petrol ether and ether in cold	Precipitation	,,	,,	Trace only
		Agglutination			Much reduced
		Complement fixation	,,	,,	Unaffected
		Protection	,,	,,	Unaffected

Merrill and Fleisher (1932) also found that rabbit agglutinins were unaffected by extraction with alcohol and ether

In general the extraction reduced the agglutinating and precipitating power of sera, while the combining and protective powers were unaffected The amount of extracted antitoxic serum that was required to prevent a skin reaction when mixed with a given

amount of diphtheria toxin and injected into the skin of rabbits was but slightly greater than the amount of unextracted antitoxic antiserum required The combination of toxin with the antitoxin of extracted serum must have been nearly as strong as with that of unextracted serum But the protective antibody of the extracted antipneumococcal serum was only partially removed by adsorption with pneumococci Some of the antibody of such sera seems to have lost the power of combining with antigen The protective power of the serum was, however, not reduced Horsfall and Goodner (1935) found that if extracted serum was injected into the peritoneal cavity of mice and the peritoneal fluid was withdrawn after 30 minutes this fluid was now able to agglutinate pneumococci Combining and agglutinating powers were therefore restored *in vivo* , they were also restored to a considerable extent by the addition of suspensions of lipins to the extracted sera (Horsfall and Goodner, 1936, c) What is even more remarkable is that the activity of extracted horse-serum was restored by the addition of lecithin (from egg-yolk), and not by cephalin, while that of extracted rabbit serum was restored by cephalin (from calf-brain) and not by lecithin Reactivation by these lipins was inhibited by the previous addition of suspensions of cholesterol The amounts of the lipins required were small—0 1 mg to 1 c c of extracted serum This is considerably less than the amount of lipin found in specific precipitates by Horsfall and Goodner (1936, a) It appears that these larger amounts that were found in the precipitates were not essential to the reaction and were merely adsorbed by the precipitate (Horsfall and Goodner, 1936, a) The essential lipin was, however, of the same order as that (2 75 per cent) found by Chow and Goebel (1935) in purified antibody, since the amount of antibody protein in 1 c c of serum is usually of the order of 10 mg Addition of a suspension of the lipins extracted from serum did not restore activity

Since the activity of antisera is restored by the addition of lipin, the loss cannot be attributed to the possible changes, not connected with loss of lipin, which the proteins undergo in the process of extraction

The restoration of activity on the addition of lecithin or cephalin may be ascribed to complex formation between antibody protein and lecithin or cephalin, comparable to the complexes studied by Schulman and Rideal (1937, a) The lipin may either affect the orientation of parts of the antibody protein molecule, thereby adapting it to antigen , or may block polar groups of the antigen-antibody complex and thereby assist precipitation The inhibitory effect of cholesterol is comparable to the inhibition of haemolysis by, for example, saponin or sodium cetyl sulphate (Schulman and Rideal, 1937, b) It is probably due to the formation of a complex between cholesterol and the activating lipin

M The Zone Phenomenon

In studying the effects of varying the proportions of antigen and antibody two procedures may be adopted (α) The amount of

antibody may be kept constant and the amount of antigen varied, or (β) vice versa The first procedure is generally employed in precipitin reactions, while the second is usual in agglutination reactions In Fig XXIII concentrations of antibody are represented vertically (diminishing downwards), and concentrations of antigen horizontally (diminishing to the right), procedure (α) follows the horizontal rows in this diagram and procedure (β) the vertical columns The results may be considered both from the point of view of the amount of precipitate or degree of agglutination and of the time required for flocculation or agglutination

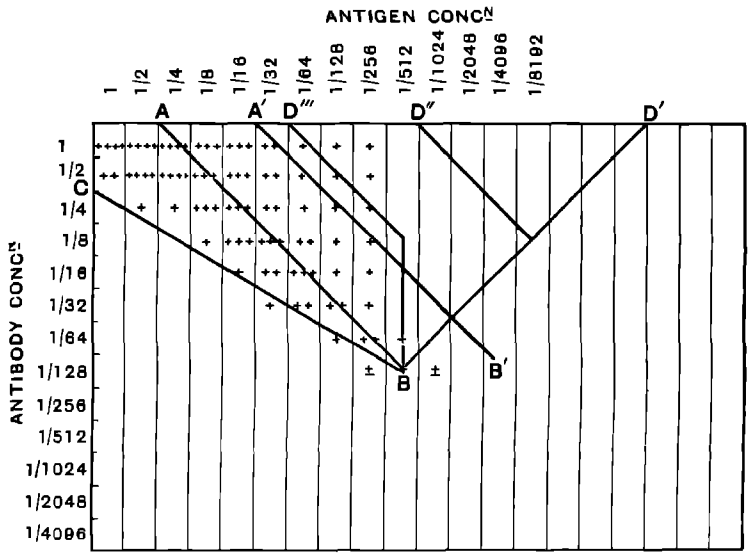


FIG XXIII Diagram of effect of varying antigen-antibody ratios on rate and amount of precipitate

1 *Procedure* α *Constant Antibody*

The type of result usually obtained in precipitin reactions is that shown in the row 1/4 With the antigen in great excess (post-zone) no precipitate forms, as the amount of antigen is reduced the amount of precipitate rises to a maximum and gradually falls again to zero (prozone)

Dean and Webb (1926) pointed out that, in such a series as here shown, flocculation appeared first in the mixture in which not more than traces of either antigen or antibody were demonstrable in the clear supernatant fluid after precipitation When the amount of antigen added was lower, antibody was detectable in the supernatant, which was clear, when the amount of antigen added was greater it could be detected in the supernatant, which was cloudy They also made dilutions of antibody up to 1/80, and added to constant amounts of each antibody dilution a series of diminishing amounts of antigen

(i.e followed the rows) They found that in every series the ratio of antigen to antibody that flocculated most rapidly was constant This ratio they called the optimum proportion In Fig XXIII the points representing such mixtures lie along a diagonal such as *AB*

Dean and Webb used horse serum as antigen , similar results may be obtained with egg albumin (Taylor, Adair and Adair, 1932), and azo-proteins (Marrack and Smith, 1931, b) Duncan (1932, a) obtained similar results, using yeast gum as antigen and serum dilutions from 1/5 to 1/20, and Smith (1932) with antisera to Type I pneumococcus and the corresponding polysaccharide In the last two cases neither antigen nor antibody was detectable in the supernatant fluid from the mixture that flocculated most rapidly

Burnet (1931, b) found most rapid flocculation of staphylococcal toxin and antitoxin in mixtures in which antibody was in excess The ratio of antigen to antibody in the most rapidly flocculating mixture was 1/6 that at the neutral point, and antibody could be detected in the supernatant fluid

However, such toxin preparations contain a mixture of several antigens and the antibody involved in the most rapid flocculation may not have been the same as that detected in the supernatant fluid

(*a*) *Composition and the amount of the precipitate*

In the earlier experiments, with antigens which could not be distinguished from the antibody protein, it was only possible to calculate the ratio of antigen to total precipitate when antibody was in excess, since only then is antigen fully precipitated The introduction of antigens which could be separately estimated, and the use of further methods, has made it possible to follow this ratio through the whole range of precipitation This has been done with great thoroughness by Heidelberger and Kendall (1929, 1935, a, b, c, and d), using as antigens S S S III, azo-protein R-salt-azo-biphenyl-azo-egg-albumin and egg albumin (fig XXIV) Somewhat similar results, less thoroughly worked out, have been obtained by Breinl and Haurowitz (1930), using haemoglobin as antigen, and by Haurowitz and Breinl (1933) and Marrack and Smith (1931, b), using azo-protein made with *p*-amino-benzene-arsinic acid and iodo-protein as antigens Haurowitz and Breinl found that measurement of antigen in the precipitate colorimetrically and by arsenic estimation gave the same result

Fig XXIV shows the relation between the amount of antigen added to a constant volume of antibody solution and the amount and composition of the precipitate formed In each case, as the amount of antigen added rises, the total amount of precipitate rises and then falls slowly, in most cases to zero, when the amount of antigen added is sufficient At the same time, the ratio of antibody protein (A)[1] to antigen (G) in the precipitate decreases

[1] Assuming that all the protein, derived from the antibody solution, that is contained in the washed precipitate is actual antibody

The curves can be divided into four zones (1) that of antibody excess, in which antibody is detectable in the supernatant fluid, (2) the equivalence zone, in which neither antibody nor antigen, or traces of both, can be detected in the supernatant, (3) the first

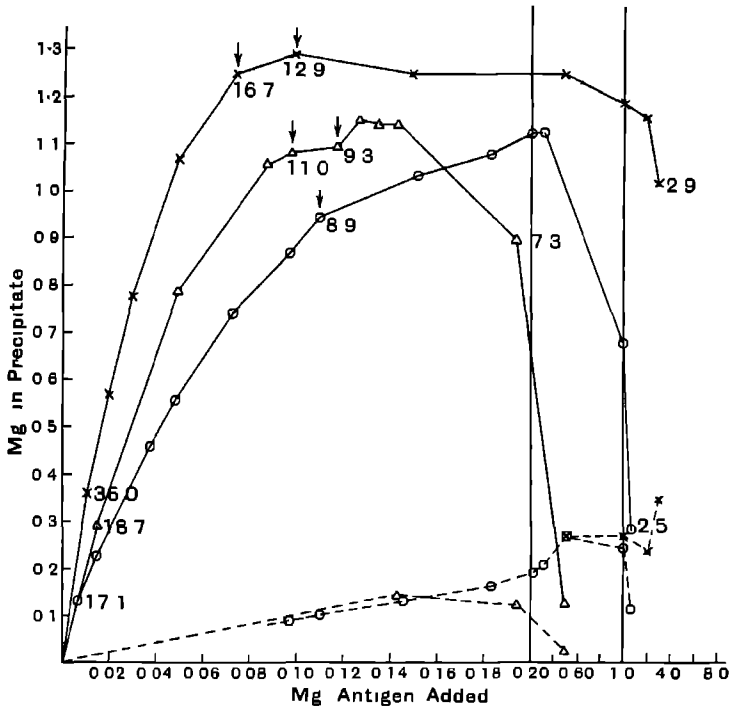


FIG XXIV Amounts of antibody N (A) and antigen in the precipitate formed from 1c c of antibody solution, on the addition of the amounts of antigen shown as abscissae

Continuous lines, antibody N, broken lines, antigen Egg albumin and azo-protein expressed in mg of protein nitrogen, S S S III in mg of polysaccharide

× S S S III and homologous antibody, B 62
○ Azo-protein and homologous antibody
△ Egg Albumin and homologous antibody 3 87 II

The arrows show the points at which neither antigen nor antibody were detectable in the supernatant fluids The figures against points on the curve show the ratios of antibody to antigen in the precipitate at these points

The scale of the abscissae is changed twice in order to bring the whole curve into the figure

From the figures of Heidelberger and Kendall (1935, b, c, d)

zone of antigen excess, before the amount of A in the precipitate begins to fall off, (4) the second zone of antigen excess (the inhibition zone, in which A remains in the supernatant fluid, in the

form of soluble compounds with antigen, but is no longer detectable as antibody [1]

In zone (1), as the amount of antigen added is increased, the amount of A in the precipitate rises as the amount of antibody detectable in the supernatant falls

In all the cases studied the protein derived from the antibody solution (A) continues to rise with progressive increase of the amount of antigen added, after antibody is no longer detectable in the supernatant fluid This is presumably due to the presence in the antibody solutions of molecules with few or weak combining bonds for antigen Such molecules can be built into an aggregate along with more active antibody molecules When antigen is added to antibody solutions in amounts insufficient to precipitate all the antibody, the more avid molecules are precipitated first and the less active molecules are left in the supernatant fluid Their presence there cannot then be detected owing to their inability to form large aggregates with antigen When larger amounts of antigen are added these weak antibody molecules are built into the aggregate formed and add to the total protein precipitated

The ratios of antibody protein to antigen in the precipitates at the equivalent point (the middle of the zone in which neither antibody nor antigen or trace of both appear in the supernatant fluid) and at the extremes of antibody and antigen excess as shown in Table XXIV

The behaviour of the diphtheria-toxin-antitoxin system is altogether abnormal The range within which precipitation occurs is very narrow and no precipitate forms in the region of *antibody* excess when the antibody-antigen ratio exceeds about twice the ratio at equivalence point (Healey and Pinfield, 1935, Pappenheimer and Robinson, 1937), precipitates will also dissolve in excess antibody, if sufficient is added (Healey and Pinfield, 1935) The haemoglobin antibody system also is abnormal as only a fraction of the antigen is precipitated, although the antigen might be supposed to be uniform, (Wu, Cheng and Li, 1928, Breinl and Haurowitz, 1930)

The reaction between crystalline urease and anti-urease rabbit serum has been studied by Kirk and Sumner (1934) They found that when a certain excess of antigen was added to a constant amount of antiserum the weight and composition of the precipitate was constant The constant composition found by Wu, Cheng and Li (1928) and Wu, Sah and Li (1929) is partly explained by the narrow range within which they worked

(b) Agglutination of Bacteria

Duncan (1932, b) also demonstrated " optimum proportions " in the agglutination of various bacteria When constant amounts of serum and diminishing amounts of antigen were used agglutination

[1] This antibody protein enters into the further precipitate that forms when more *antibody* is added to the supernatant fluid (Heidelberger and Kendall, 1935, b)

occurred most rapidly with a certain ratio of antigen to antibody, which remained constant with antiserum dilutions ranging from 1/9 to 1/144 Miles (1933) obtained similar results

TABLE XXIV

Ratio of Antibody to Antigen in precipitates formed in Equivalence Zone and at Extreme Antibody and Antigen excess

Antigen.	*Molecular Weight*	*Antibody Excess*	*Equivalence Zone*		*Antigen Excess.*
			Observed	*Calculated* (F_1)	
S S S III (A_1)	4 000	230	50 to 110	58	19
Diphtheria Toxin[1] (H)		6 8	3 4		—
Egg Albumin (A_2)	34 500[2]	21 5	8 6 to 15	13 4	3 5 to 7 5
,, ,, (B)	idem	15 8	9 8 to 16 8	13 4	—
,, ,, (C)	idem	17 3	9 2 to 12 3 8 to 18	13 4	—
Haemoglobin (D)	68,000[2]	—	10	9 7	—
Serum Albumin (C)	70,200	14 5	6 3 to 7 5	9 7	—
Serum Pseudo-Globulin (E)	(?) 103,800[2]	9 5	3 2 to 4 5	7 3	—
Fulgur (Busycon) Haemocyanin (F_2)	4,960 000[2]	—	0 59 to 0 75	0 97	—
Limulus Haemocyanin (F_1)	1,200,000 2,500,000 (300,000)	—	0 91 to 1 40	1 75 1 33	—
Azo-protein (A_2)	—	17 1	8 3 to 13		2 5
,, (G)	—	—	1 6 to 8 5		—
Iodo-Protein (G)	—	—	4 55 to 6 8		—

A_1 Heidelberger and Kendall (1935, b)
A_2 idem idem (1935, c)
A_3 idem idem (1935, d)
B Culbertson, (1932)
C Taylor, Adair and Adair (1934)
Dean, Taylor and Adair (1935)
D Wu, Cheng and Li (1928)
E Marrack and Smith (1931, a)
F_1 Boyd and Hooker (1934)
F_2 idem idem (1936)
G Marrack and Smith (1931)
H Pappenheimer and Robinson (1937)

[1] Assuming that pure diphtheria toxin contains 0 00046 mg of nitrogen per Lf
[2] These molecular weights are those used by Boyd and Hooker in the calculation of column 5, not the more recent values given in Table III This does not, however, affect the calculation greatly

2 *Procedure β Constant Antigen*

It is obvious from the diagram (Columns, Fig XXIII) that with a sufficiently concentrated antigen (e g column 1/2) there will be no zone of antibody excess (prozone) in which precipitation does not take place When, however, the antigen is not too concentrated the presence or absence of such a zone will depend on the boundary of precipitation on the right hand of the diagram Sobotka and Friedlander (1928) found that with some antipneumococcus sera the lowest concentration of polysaccharides at which visible precipitation occurred was such that the product (antigen concentration)

(antibody concentration) was approximately constant That is, the right-hand limit of precipitation lay along a line such as *BD'* (Fig XXIII) In such cases no prozone would appear However, some antipneumococcal sera (Friedlander, Sobotka, and Banzhaf, 1928) gave right-hand limits lying along lines sloping like *BD"* and *BD'''* In such cases a prozone would appear with the lower antigen concentrations

Taylor (1931) found that when the antigen (horse-serum protein) was kept constant β procedure, the most rapid flocculation did not occur with the ratio antigen/antibody which flocculated most rapidly when α procedure was used , but that flocculation became more rapid with mixtures containing up to twice this ratio However, it is apparent from the figures of Dean and Webb (1926) that there was some slowing off with higher antibody concentrations , this was also found in some cases by Eagle (1932) Recently Taylor (1933), using crystalline egg albumin as antigen, has shown that the most rapid flocculation with the β procedure occurs with a ratio antigen/antibody from 0 75 to 0 57 of the ratio at which most rapid flocculation occurs with the α procedure When a still greater excess of antibody was added flocculation was slower, but precipitation eventually occurred even with great antibody excess

Duncan (1932, a) found that with the β procedure using yeast gum as antigen the most rapid flocculation occurred with an antigen/antibody ratio 1/8 of that which gave the most rapid flocculation with the α procedure , an excess of antibody was found in the resulting supernatant fluid

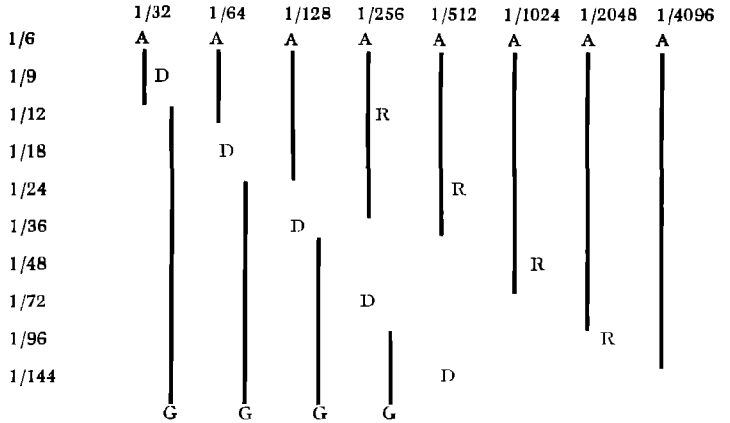
Duncan (1932, b) also found that in the agglutination of bacteria the most rapid agglutination using β procedure (the usual method) occurred at a ratio of antigen to antibody 1/6 of the ratio for most rapid agglutination using α procedure (see Table XXV) Miles (1933) found that in the agglutination of the Brucella group the ratio was in the region of 1/4 The points representing the most rapidly flocculating mixtures by procedure β, therefore, lie along a line *A'B'* in the diagram, parallel to *AB* but farther to the right Bier (1931) found that high salt concentration shifted the most rapidly agglutinating mixture towards antibody excess

In the Ramon method, with diphtheria toxin and antitoxin, β procedure is used Diminishing quantities of antitoxin are added to a constant amount of toxin Nevertheless, the mixture at which flocculation occurs most rapidly is approximately balanced Neither the precipitate nor the supernatant contains excess of toxin or antitoxin According to Timmerman (1934), the antibody to antigen ratio found by the α method is 10 to 15 per cent lower than that found by the β method In this case the range of flocculation is very narrow , the highest ratio of antibody to antigen which will give a precipitate is only some four times the lowest It is probable that the ratios of most rapid flocculation by either procedure are brought close together

A prozone is frequently observed when the agglutination of bacteria is performed in the usual way with the amount of antigen constant

TABLE XXV

Most rapidly flocculating mixtures by α procedure = D, and by β procedure = R, in agglutination of a B enteritidis *suspension by Salmonella "Dublin" serum* (Duncan, 1932, b)



The vertical lines A A, A indicate that agglutinin was present in the supernatant after flocculation, the lines G, G G that agglutination was not complete

Shibley (1929) considered that this only occurred with old or heated sera. He confirmed Streng's (1909) observation that some agglutinating sera, when moderately heated, would no longer agglutinate in the higher concentrations (Table XXVI), although the highest dilution at

TABLE XXVI

Prozone produced by moderate heating of antiserum to B dysenteriae (*Shiga*) C = *complete agglutination* (Shibley, 1929)

Antiserum heated for 10 minutes at	*Dilution of serum*								
	1/20	*1/40*	*1/80*	*1/160*	*1/320*	*1/640*	*1/1280*	*1/2560*	*1/5120*
63° C	C	C	C	C	C	C	C	C—	—
64° C	±	±	C	C	C	C	C	C—	—
65° C	—	—	C—	C	C	C	C	C—	—
66° C	—	—	—	—	C	C	C	C—	—
69° C	—	—	—	—	C	C	C	C—	—
70° C	—	—	C—	C	C	C	C—	±	—
71° C	C—	C—	C	C	C	C	±	±	—
72° C	C	C	C	C	C	C—	—	—	—
74° C	C	C	C	C	C	C—	—	—	—
76° C	—	—	—	—	—	—	—	—	—

which agglutination occurred was unaltered by this amount of heating. Further heating abolished this prozone and reduced the titre of the serum. Shibley centrifuged off bacteria which had been mixed with high concentrations of moderately heated serum, and had

not agglutinated, these bacteria were not agglutinated when resuspended in fresh agglutinating serum Some of the antibody appears to have been so changed that, although it would combine with the antigen, the second stage, the change from hydrophil to hydrophobe, would not occur, at any rate when antibody was in excess

Duncan (1937) did not find a prozone in the agglutination of flagellated intestinal bacteria With smooth non-flagellated bacteria a wide prozone of incomplete agglutination was found in high and low salt concentration, for example, with *B dysenteriae* up to a 1/64 dilution of antiserum in 2 3 M NaCl, and to a 1/2048 dilution in 0 0044 M NaCl The prozone was narrowest in 0 29 M NaCl, that is, in a salt concentration about double that commonly used

3 *Stability of antigen-antibody aggregates*

A third method of approach is to shake up the precipitate or agglutinated bacteria formed in a series of antibody-antigen mixtures and to find which of these is least easily dispersed Brown (1935) has called this the γ procedure, she found that in the system S S S I or II and antisera, the ratio given by the γ procedure corresponded closely with that given by the α procedure On the other hand Duncan (1934, b) studying the agglutination of smooth non-flagellated *B dysenteriae* Flexner found the β and γ procedures gave the same ratio

4 *Effect of Salt Concentration, Hydrogen ion Concentration and Temperature on Optimum Proportions*

According to experiments of Dean and Webb (Dean, 1931) the optimum proportions (α method) of antigen to antibody were unaltered in concentrations of NaCl between 0 2 and 1 per cent, in concentrations above 1 per cent the ratio was changed Brown (1935) also found that the optimal proportions of antibody to antigen (S S S I, α method) was higher in 2 and 3 per cent NaCl than in one per cent Duncan (1934) observed a similar shift of the ratio by the β procedure, using a suspension of a smooth, non-flagellated strain of *B dysenteriae* Flexner as antigen, the ratio at optimum proportions by the α procedure was not affected by high salt concentrations In low salt concentrations (e g 0 1 per cent) Duncan found that the optimum proportion, by either method, was shifted towards a lower antibody-antigen ratio

Brown (1935) found that the ratio of antibody to antigen S S S I at optimal proportions might be increased with a fall of pH from 8 0 to 6 73 When the pH was reduced to 6 0 or lower the increase of the ratio was more striking (e g from a ratio of 1 c c of antiserum to 0 29 mg of S S S I at pH 8 0 to a ratio of 1 c c antiserum to 0 19 mg of S S S I at pH 6 0)

Using purified diphtheria toxoid and antitoxin Watson and Longstaff (1926) found that the ratio of most rapid flocculation (β procedure) was unchanged between pH 6 0 and 10 0, although flocculation was much delayed in the more alkaline solutions

Bayne-Jones (1928) and Schmidt (1930, c) also found that the ratio of antitoxin to toxin of the most rapidly flocculating mixture was unaffected within a moderate range of pH (6 4 to 8 4), outside this range the results were irregular

Temperature changes between 0° and 40° C do not appear to affect the α procedure ratio in the systems human or dog serum antigen and rabbit serum antibody, and the system diphtheria toxin and antitoxin On the other hand with S S S I and antibody, α procedure, flocculation at 0 4° C occurs first in the mixture containing the largest amount of antigen (Brown, 1935)

It is difficult to attempt any explanation of the effect of physical environment on optimal proportions without further quantitative investigations It is natural to correlate the shift of the optimum antibody-antigen ratio of one pneumococcal polysaccharide (S S S I Brown, 1935) with the decrease of antibody protein precipitated in the higher salt concentration by S S S III (Heidelberger, Kendall and Teorell, 1936), and the dissociation of antibody from precipitates of S S S I or II and antibody (Heidelberger and Kendall, 1936) This may also be correlated with the change of physical state of polysaccharides with rising salt concentrations (Heidelberger and Kendall, 1932, a and c) The amount of antibody precipitated by egg albumin is little affected by high salt concentration, but the optimum proportions in a similar system, serum albumin and antibody, are shifted in 2 and 3 per cent sodium chloride solutions

Bier (1931) and Duncan (1934) suggest that the shift of the β optimum of agglutination of bacteria may be due to the effect of salt concentrations on cohesion of the bacteria However, cohesion between smooth non-flagellated intestinal bacteria must be relatively weak as they do not agglutinate in low salt concentrations although their surface potential is very low The shift in optimum proportions cannot be attributed to changes in the amount of antibody bound, as this is little affected by salt concentration

N The Zone Phenomenon in relation to the Nature of the Mechanism

The remarkable point about the zone phenomenon is the difference between the results of antigen and antibody excess Excess of antigen produces inhibition whichever procedure is used Excess of antibody does not constantly produce inhibition in β procedure, and only begins to slow up flocculation when in considerable excess If it is supposed that antibody is denatured on combination with antigen, this difference between the effect of excess antibody and excess antigen would be accounted for on the supposition that antibody was denatured while antigen was not With antigen in excess the complex formed would be coated and protected by undenatured antigen The slowing up of the reaction with antibody excess in β procedure would be attributed to less complete denaturation of antibody when in excess

On the supposition illustrated in Fig XXII this difference between the effects of excess of antigen and antibody would be attributed

primarily to the presence of more combining sites on antigen than on antibody As a result the antigen-antibody precipitate would be mainly composed of antibody This would mean that in the diagram of Fig XXII antibody molecules pack tightly round antigen molecules and their polar groups neutralize each other, even when antibody is in excess They will therefore be aggregated as relatively hydrophobe suspensions even when not bound by specific bonds On the other hand, with antigen excess, as in C or E in Fig XXII, no packing of the molecules on the surface occurs as there are not receptor sites on the antibody molecules to hold more antigen, the polar groups are available to attract water and flocculation does not occur

Considering α procedure from this point of view, no precipitation will occur in the post-zone (antigen excess) owing to the formation of small aggregates, such as C or E in Fig XXII, protected by the excess of antigen With smaller quantities of antigen continuously larger aggregates will be formed, possibly arranged as a lattice with incomplete packing of the antibody molecules These may settle, leaving the supernatant fluid cloudy, owing to the presence in it of large aggregates At optimum proportions a continuous lattice will be formed When the antigen concentration falls below this level aggregation will be slower, mainly owing to the reduction in the number of antigen molecules which serve as centres of aggregation, also to some degree because the formation of a continuous lattice fails progressively more and more as no further antigen molecules are available to provide links for the formation of larger structures when aggegates of moderate size, such as D, are formed Also some polar groups on the surface of particles such as D, may not be blocked, and therefore retain their attraction for water, and to some extent inhibit the clumping of the particles as a hydrophobe suspension

Hooker and Boyd (1935) have found, with several protein antigens, that in the zone of antibody excess, when the amount of antigen is 1/3 to 1/4 or less of the amount equivalent or optimal to the antibody present, the rate of flocculation is proportional to the concentration of antigen This is in agreement with von Schmoluchowski's formula for slow flocculation, when the fraction of collisions between particles resulting in union is constant That is, when this degree of antibody excess is reached the primary antigen-antibody units cease to differ in their attraction for one another

When β procedure is used the results will be the same up to the point at which a continuous lattice can be formed Beyond this point the chief reason for the slowing up in α procedure will not take effect, since the antigen concentration is constant, and the reaction will be accelerated by the increased antibody concentration the most rapid flocculation will therefore come at some later point when the progressively smaller size of the aggregates which can be formed balances the increasing rate of their formation

In connexion with the prozone which may be found in β procedure it is necessary to take into account factors which will vary with the varying antiserum concentration First there is the effect

observed by Streng (1909) and Shibley (1929) in moderately heated sera It appears that the occurrence of a prozone, when these heated sera were used, was due to the presence of modified antibody, which was preferentially adsorbed by antigen but would not produce agglutination This modified antibody was removed by passing through a Berkfeld N filter or by shaking with talc Presumably, as a result of the partial heat denaturation of antibody protein, a partial loss of affinity for water had been produced In consequence the modified antibody was more readily adsorbed by a filter or by talc, and also would be more adsorbed by the antigen if its specific binding sites were undamaged But the further binding up of the antigen-antibody complex into a stable structure may have been impossible either because the polar groups of the antibody molecules were no longer available or because the molecules had already aggregated in an unsuitable manner

On this view the prozone would be due to the presence of modified antibodies (agglutinoids) The work of Miles (1933), however, shows that this is not the whole explanation For when dilute antigen suspensions were used (β procedure), a most rapidly agglutinating proportion of antiserum to antigen was found , when the amount of antiserum was increased the time of agglutination rose first and then fell again If the presence of agglutinoid was the sole reason for the delay of the reaction observed when first the amount of antiserum was increased, the later acceleration should not have occurred

The concentration of non-specific proteins also varies in β procedure owing to the variation in the amount of antiserum added The effect of non-specific proteins on the amount of precipitate, found in some cases (p 143), is doubtless due to adsorption of a thin layer on the antigen-antibody aggregates Such a layer may add very little to the total volume of *aggregate*, since the covering of only one globulin molecule by a layer about 4 Å thick would only increase its volume by about 40 per cent But the precipitation of more stable suspensions of antigen-antibody aggregates, for example, when antigen is in excess, may be assisted by such a layer , the effect being similar to the aggregation and sedimentation of red blood corpuscles by globulin

It is possible that the results of β procedure are always considerably affected, especially when antibody is in excess, by these factors, These would account for the varying slope of the line *BD* (Fig XXIII) When α procedure is used these factors are constant throughout

According to the point of view suggested, the optimum proportion (α procedure) is considered to be that ratio of antibody to antigen which is capable of forming a stable lattice [1] This will be determined partly by a question of size In the instance in which the antigen, serum globulin, is of approximately the same size as the antibody the ratio is 1 to 4 This would be given by a lattice such as shown in Fig XXII (but in three dimensions), with each antigen molecule

[1] Although the mode of approach is different, the conclusions arrived at here are very similar to those of Taylor (1931) and Burnet (1931 a)

surrounded by 16 antibody molecules, and each antibody molecule by 4 antigen molecules (not three as in the flat figure) It is not necessary that, at the proportion that forms a stable lattice, all the " receptor sites " on the antibody should be satisfied by " determinant " antigen groups But, in all cases in which there is reason to suppose that a single antigen is involved, most rapid flocculation occurs in the equivalence zone (in which, even if all the antibody and antigen are not built into the precipitate, no excess of either is present in the supernatant)

Boyd and Hooker (1934, 1936) have suggested that at the equivalence point one molecule of antigen is covered by as many antibody molecules as can be simultaneously brought into contact with its surface without deformation They assume that the antibody globulin molecule is formed of a group of three equal spheres of weight 34,500 all three of which are in contact with the antigen and not appreciably distorted They derive for the ratio of antibody to antigen at equivalence the formula

$$\frac{34{,}500\left[2 + \dfrac{90}{\tan^{-1}\sqrt{\tan\left(\dfrac{3\theta}{2}\right)\tan^{3}\left(\dfrac{\theta}{2}\right)}}\right]}{\text{Molecular weight of antigen}}$$

Where $\rho = \sqrt[3]{\dfrac{34{,}500}{\text{Molecular weight of antigen}}}$

and $\sin\theta = \dfrac{\rho}{1+\rho}$

Ratios calculated from this formula are shown in Table XXIV They agree satisfactorily with the ratios found It may be questioned, however, whether it is so justifiable to assume that the molecule of S S S III is a sphere of molecular weight 4,000 There seems to be no doubt that the relative sizes of antibody and antigen molecules must have considerable effect in determining the ratio at optimum proportions on the equivalence point But this ratio is probably affected also by the number of combining groups on both antibody and antigen , otherwise the ratio should be constant for any particular system The author has found (unpublished) that with a given antiserum to *p*-amino-benzene-arsinic acid, the amount of antigen equivalent to a given volume of antiserum at optimal proportions was inversely proportional to the arsenic content of the antigen

In the agglutination of bacteria the formation of a lattice would be of considerably less importance, and therefore the increased rapidity of agglutination at optimum proportions considerably less marked

We have noticed a difference between the agglutination of some bacteria and the formation of precipitates by molecularly dispersed antigens Deposits of agglutinated bacteria are most stable at the β ratio while precipitates are most stable at the α ratio It is possible that the actual mechanism involved differs in this respect ,

molecularly dispersed antigens are built into a complex as suggested, whereas the agglutination of bacteria takes place in two stages (1) the formation of molecular aggregates on the surface of bacteria with loss of free polar groups and (2) the agglutination of bacteria as hydrophobic particles This second process would be non-specific

Pneumococci take up a much larger amount of antibody than do other bacteria and may, therefore, unlike other bacteria, be bound together specifically—hence possibly the difference between the results of Topley, Wilson and Duncan (1935) on the one hand, and Abramson (1935), Hooker and Boyd (1936, a) on the other

In this connexion it is interesting to consider the minimum amount of antibody globulin necessary to produce agglutination of bacilli Supposing that the bacilli are 3μ long and 0 4μ across and that there are 10^9 per c c , the minimum amount of protein required to form a continuous layer of the minimum thickness found by Hughes and Rideal (1931) would be about 10^{-6} g per c c , or 0 0001 per cent Now it is claimed that agglutinin solutions can be prepared which will not give reactions for proteins (given by solutions containing more than 0 001 per cent of protein), or contain less than 0 002 per cent of protein, and yet will agglutinate typhoid bacilli when diluted 1/1,000 (Ottenburg and Stenbuck, 1923–4) The 1/1,000 dilutions cannot contain enough protein to cover more than 1/50 of the surface of the bodies of the bacilli, much less that of the flagella The theory that ascribes the hydrophobe character of sensitized bacilli to the formation of a layer of denatured antibody globulin, demands that an appreciable fraction of the bacillary surface should be covered It appears that agglutinins can be obtained which do not contain sufficient protein to do this On the other hand, if it is only necessary to provide links of antibody globulin between the bacilli in order to produce agglutination, much less is required, if the affinity of the antibody for the bacillary surface is strong

Heidelberger and Kendall (1935, a, b, c, d,) have put forward a theoretical consideration of the quantitative aspects of the precipitin reaction which is based on five assumptions

(1) Antibody is considered to be a protein which may be accurately estimated through the determination of nitrogen in the washed specific precipitate [1]

(2) Antigen and antibody are multivalent with respect to one another

(3) Combination of antigen and antibody proceeds by a series of bimolecular reactions, the compounds first formed are soluble, owing to the multivalence of antibody and antigen combination continues until large aggregates are formed, these settle out

(4) The dissociation of compounds first formed is negligible

[1] The specific precipitate may contain an appreciable amount of lipin nitrogen, particularly in the region of extreme antibody excess (Horsfall and Goodner, 1936 a) This does not affect the theory and allowance for this lipin nitrogen improves the agreement of experimental data with theory

(5) When antigen is in considerable excess a soluble compound is formed in which the ratio of antibody to antigen is about half that at the equivalence point

A high degree of irreversibility appears to be essential since the ratio of antibody nitrogen to antigen in the precipitate depends on the relative proportions of antigen and antibody present in the reacting mixture and not on their final concentrations in the supernatant fluid

In the simplest form antibody (a) and antigen (g) may be supposed to be bivalent In the zone of antibody excess the bimolecular reactions may be supposed to take place in stages ,

$$\begin{aligned} &\text{1st stage} && a + g \longrightarrow ag \\ &\text{2nd stage} && ag + a \longrightarrow aga \\ &&& ag + ag \longrightarrow ag\,ag \end{aligned}$$

The " ag ag " molecule then polymerizes with " aga " or with other " ag ag " molecules to form large complexes whose composition lies between that of " aga " and " $a_n g_n$ " As the compounds once formed are irreversible, the final composition of the mixed complexes depends on the rate at which " aga " and " ag ag " are formed Heidelberger and Kendall calculate for the zone of antibody excess that if the number of antibody molecules in the reacting mixture is A, the number of molecules of antibody, y, precipitated when x molecules of antigen are added is given by the equation

$$y = 2x - \frac{x^2}{A} \qquad \longrightarrow \quad (1)$$

(A similar, but more complex equation is derived when antibody and antigen are trivalent) Antigen and antibody will be wholly precipitated when $x = y = A$ At the equivalence point the molecular composition of the precipitant would therefore be " $a_n g_n$ " However, there is good reason to suppose that the compound formed at the equivalence point does not contain equal numbers of antigen and antibody molecules and should be represented by the formula " $a_m g_n$ " For one thing, if the formula were " $a_n g_n$ ", the composition of the precipitate at the equivalent point should be constant

Heidelberger and Pedersen (1937) have found that the molecular weight of the antibody to egg albumin is that of ordinary serum globulin—about 150,000 , if the molecular composition of the precipitant at the equivalence point was $a_n g_n$ the ratio of antibody to antigen in the precipitant would be about 5/1 instead of 11/1 as actually found

Heidelberger and Kendall attempt to get over this difficulty by supposing that if the compound at the equivalence point is represented by " ag_n ", the first stage of the reactions, in the antibody excess zone, consists of a *series* of *bimolecular* reactions giving rise to the compound " ag_n ", *before* the second stage, in which occur the reactions

$$\begin{aligned} &ag_n + a \longrightarrow a_2 g_n, \\ \text{and } &ag_n + ag_n \longrightarrow ag_n,\ ag_n \end{aligned}$$

This amounts practically, to the occurence of a multimolecular reaction

$$a + ng \rightarrow ag_n$$

before the bimolecular reactions

$$ag + a \rightarrow aga,$$
$$ag + ag \rightarrow ag, ag.$$

which is improbable

This appears to be a serious objection In reversible reactions, in which the final result is independant of the order in which the reactions involved take place, the time relationship can be ignored But this theory appears to involve a high degree of irreversibility, the order in which the reactions occur is therefore essential It is, however, questionable whether compounds such as ag, ag, aga are actually irreversible

When antibody is in excess the final aggregates will have no free antigen bonds, such an aggregate can combine with more "a" only if it breaks up E g —

$$ag\ ag\ ag\ a + a = ag\ ag + aga + a$$
$$= ag\ aga + aga$$

If the combination is irreversible this should not take place Similarly the final aggregate in the zone of antigen excess should be incapable of combining with more antigen In the zone of antibody excess this point can be tested with the antigen-antibody systems considered, in the system diphtheria-toxin-antitoxin the final aggregate did combine with more antibody (Healey and Pinfield, 1935) In the zone of antigen excess also the final aggregates can combine with more antigen, as is shown by the solubility of the precipitates in excess of antigens such as S S S III and egg albumin

Supposing that equation (1) is still applicable, although antigen and antibody do not combine in equimolecular proportions at the equivalence point, it becomes

$$y = 2\,Rx - \frac{R^2x^2}{A} \qquad \longrightarrow (2)$$

when x and y are expressed in milligrams instead of in molecular weights

R is the ratio of antibody to antigen at the equivalence point This equation can also be expressed in the form

$$r = 2R - \frac{R^2x}{A} \qquad \longrightarrow (3)$$

where r is the ratio of antibody to antigen in the precipitate formed when x milligrams of antigen is added This is a simple linear equation containing two constants which measure essential properties of the antibody solution—the amount of antibody present and the combining power of this antibody

The actual experimental data obtained with S S S III and antibody fit this equation well (Fig XXV) R and A in this instance are the values, not at the equivalence point, but at the antibody excess

end of the equivalence zone, this, however, may be explained by the presence of easily dissociated antibody which may be supposed to play little part in the reaction when antibody is in excess

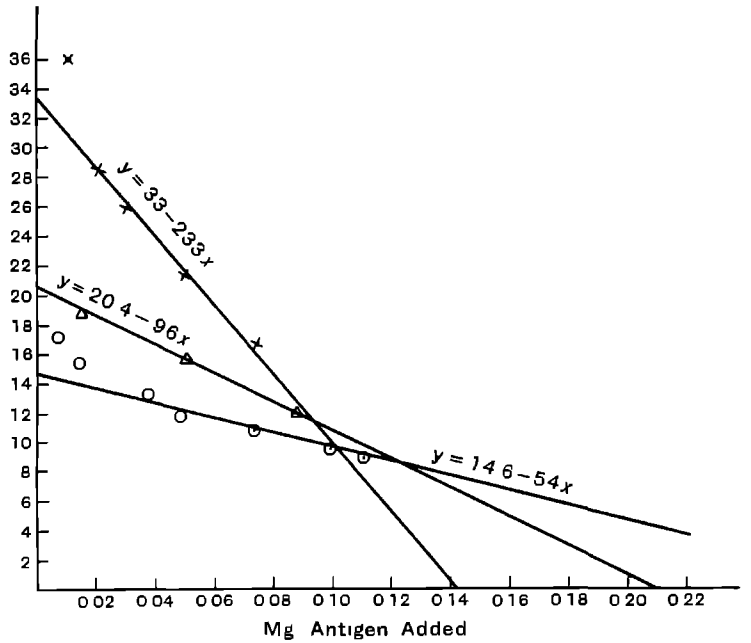


FIG XXV The lines

$$y = 2\,R - \frac{R^2}{A}\,x$$

× S S S III and antibody, B 62
○ Azo-protein and homologous antibody
△ Egg albumin and homologous antibody 3 87 II

From the figures of Heidelberger and Kendall (1935, b, c, d)

Experimental data found with azo-protein and egg albumin as antigens conform less well to this equation In the first place, the values of A and R that fit the equation are those found at the antigen excess end of the equivalence zone, although even more easily dissociated antibody is found in these sera than in the antipneumococcal sera In the second place the data fit better to a purely empirical equation

$$r = 3\,R'' - 2\sqrt{\frac{(R'')^3 x}{A}} \qquad \longrightarrow (4)$$

in which A = the maximum precipitable antibody, and R″ is the ratio at the point of maximum antibody precipitation In the case of egg albumin values of R″ which fit this equation do not agree well with those found experimentally

In the zone of antigen excess, before the inhibition zone is reached, similar equations, with the positions of antigen and antibody reversed, are applied

When excess of antigen rises above a certain level the amount of antibody precipitated begins to fall off until eventually no precipitate is formed The ratio of the amount of antigen required to produce complete inhibition to that at equivalence point (antibody remaining constant) varies greatly with the system considered, being about 100 with S S S III, 15 with azo-protein, and 8 with egg albumin In the inhibition zone Heidelberger and Kendall (1929, c, 1935, b, c) found that the ratio of antigen in solution to antibody in solution (that is, difference between total antibody and antibody precipitated) was constant both for the S S S III and azo-protein systems This fits the supposition that a soluble compound $a_m g_{n+1}$ is formed by the combination of 1 molecule of g with a slightly soluble compound $a_m g_n$ According to the law of mass action, as long as some precipitate of $a_m g_n$ is present

$$\frac{[a_m g_n]\ [g]}{[a_m g_{n+1}]} = K \qquad \longrightarrow (5)$$

$[a_m g_n]$ the concentration of the slightly soluble compound in solution is constant and small compared with $[a_m g_{n+1}]$, the concentration of soluble compound, $[a_m g_{n+1}]$ is, therefore, approximately equal to the concentration of antibody in solution,

So that

$$\frac{\text{Antigen in solution}}{\text{Antibody in solution}} = K' \qquad \longrightarrow (6)$$

Sobotka and Friedlander (1928) found that, with some sera, the product of the concentration of a solution of pneumococcal polysaccharide (S S S I and S S S II) and the minimum concentration of antibody with which it would give a precipitate was constant They suggested that this could be compared to a solubility product, an insoluble compound of antibody and polysaccharide being formed according to the law of mass action This is the inverse of the relation found by Heidelberger and Kendall However, the concentrations, the product of which should be constant under such conditions should be those of free antibody and antigen in the supernatant fluid after precipitation has occurred, not those in which the reagents are mixed Without knowing the amounts of antibody and antigen precipitated it is impossible to draw conclusions from this observation of Sobotka and Friedlander

Apart from their purely theoretical treatment, Heidelberger and Kendall have accumulated a mass of information bearing on the quantitative relationships of the precipitin reactions They have in particular demonstrated the non-uniformity of the antibody molecules For example, in serial experiments, after much of the antibody of an antipneumococcal serum has been precipitated by successive additions of small amounts of S S S III, a point is reached

at which an appreciable amount of S S S III occurs in the supernatant fluid in the presence of a concentration of antibody protein which would precipitate S S S III in a dilution of 1/10,000,000 if the antibody remaining had the same properties as the original antibody A point not made by Heidelberger and Kendall is that, when less than half of the total antibody to S S S III or azo-protein has been precipitated by serial additions of small amounts of antigen (*J exp Med*, Table II on p 567 of Vol 61 and Table III on p 476 of Vol 62), the antibody remaining in solution does not behave as did the original antibody The amount of protein precipitated by a given amount of antigen was less than would be expected from the amount of antibody left in solution

In the various fractions prepared by Chow and Goebel (1935) from one antiserum to Type I pneumococcus, the ratio of antibody proteins to antigen at the equivalent point appears to have varied considerably, these fractions may, however, be considered artefacts

In antisera to the azo-protein and egg albumin a considerable portion of the antibody was not precipitated by serial additions of small amounts of antigen, but was precipitated if sufficient antigen was added in one piece It is suggested that this fraction consists of antibody molecules with so few binding groups that they cannot form large aggregates when they are mixed with antigen in the absence of other antibody molecules They can, however, be built into the aggregate formed by antigen and more reactive antibody molecules Other work of Heidelberger, Kendall and Scherp (1936) has shown that some of the preparations of pneumococcal polysaccharides are not uniform, and it is found that a fraction of the azo-protein antigen, varying with the antiserum used, is not precipitable by antibody In their theoretical discussion Heidelberger and Kendall have assumed that it is permissible to regard antibody molecules as behaving statistically as if they were homogeneous, although allowance is made for the less reactive antibody of antipneumococcal sera by taking for A and R, in the equation (3), the values at the beginning of the equivalent zone It seems possible that the quantitative relations found may be an expression solely of this non-uniformity of antibody and antigen A much wider equivalent zone is found with the non-uniform antigens S S S III and azo-protein than with crystalline egg-albumin

Owing to the fewness of their combining groups and to spatial considerations, simple haptene molecules, which are not themselves aggregated, cannot take the place of antigen molecules in the antibody-antigen lattice Their inhibiting action on flocculation can therefore be compared to the inhibition of crystallization, from supersaturated solutions, by dyes which are adsorbed on the crystal surface (Marc and Wenk, 1910) Such adsorbed molecules do not take the place of the essential constituents of the crystal in the crystal lattice, but inhibit crystallization This affords an explanation of an unpublished observation of the reviewer that "simple haptenes", in amounts insufficient wholly to prevent flocculation,

do not alter the " optimum proportions " of antigen to antibody That is, on the present theory, they do not alter the proportions of antigen and antibody required for building a continuous lattice, although they slow the reaction by forming surface films

Possible explanations of various other points suggest themselves

The decreased reversibility on standing may be due to increased building up into a uniform structure, together with the replacement of intermolecular attraction by actual chemical bonds

Temperature can affect immunity reactions in two ways, apart from actual damage to antigen or antibody at temperatures above 55° C In the first place, a rise of temperature will increase the number of effective collisions between antigen and antibody, and will therefore accelerate the reaction In the second place, increased heat vibration will tend to break down the compound of antigen and antibody and complexes built up by them Hence immunity reactions take place more rapidly at higher temperatures up to a certain limit, but combination becomes less firm and may break down at the higher levels

O The Relation between free and combined Antibody, Freundlich's Isotherm and the Law of Mass Action

The relation between free and combined antibody has been studied mainly by using particulate antigens, which can be centrifuged off, leaving a supernatant fluid in which the remaining antibody can be measured The relation commonly found is shown in Fig XXVI Such results were found, for example, by Eisenberg and Volk (1902, absorption of agglutinin by bacteria) and Cromwell (1923, absorption of haemolysin by red blood corpuscles) When small amounts of antibody are added practically all is adsorbed, as the amount added increases the ratio of bound to free falls, giving a curve concave to the horizontal axis But many more than 1,000 times the minimal agglutinating or haemolytic dose may be bound without a limit being reached A different type of result was obtained by Dreyer and Douglas (1910), the amount of agglutinin adsorbed by bacteria rose to a maximum and then with the addition of increasing amounts of antibody fell off, in some cases to nothing Similar results were obtained by Manwaring (1905) using haemolysin Cromwell (1923) considered that some haemolytic sera contained what may be called haemolysinoids For red blood corpuscles which had been exposed to a serum from which the haemolysin was not readily absorbed, and had been centrifuged off, took up fewer haemolytic doses when mixed with a new lot of haemolysin, than did cells which had been exposed to a readily absorbed serum, in spite of the fact that the latter had taken up a larger number of units at their first exposure This suggested that the serum from which the haemolysin was not readily absorbed contained some substance which combined with the red blood corpuscles and prevented the absorption of haemolysin, but which itself would not haemolyse cells Eisenberg and Volk (1902) supposed that agglutinoids, which would combine with bacteria more

readily than agglutinins, but would not agglutinate them, accounted for anomalous results with bacteria Dreyer and Douglas, however, showed that this did not account for their curious result, as sera from which the agglutinins had been partially absorbed by bacteria still showed the phenomenon Had the phenomenon been due to agglutinoids which were preferentially absorbed, the first lot of bacteria should have removed them

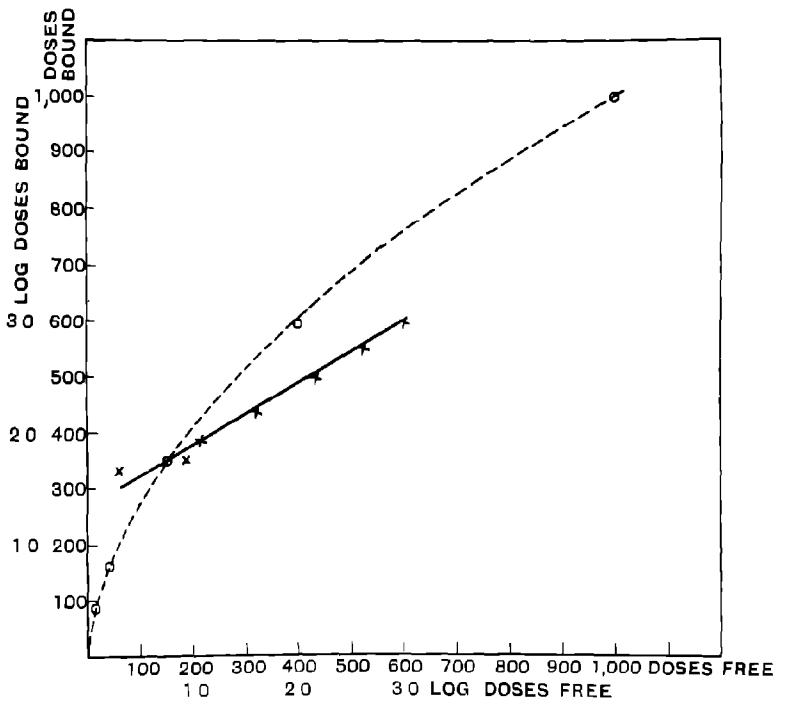


FIG XXVI Relation of haemolysin bound to haemolysin free, broken line, and of log (haemolysin bound) to log (haemolysin free), continuous line (Cromwell, 1923)

The following section on titration of antibodies suggests a criticism of all these experiments When a large proportion of the antibody in the added serum is absorbed, the proportion of non-specific protein in the residual fluid which is to be tested will be much increased, and the results of the titration may well be affected thereby This, however, will not account for the results of Dreyer and Douglas and similar results of Manwaring (1905), which are difficult to account for on any hypothesis

In these investigations complex particulate antigens have been used owing to the ease with which they can be centrifuged off They are, however, open to the objection that they contain several antigenic

substances, and that the antisera may contain antibodies to these. These antibodies would not be distinguished, and measurements of absorption of antibody would not be measurements of the absorption of a single substance

Also, the assumption is usually made, without any warrant, that the antigen molecules or particles and the antibody molecules in a given reaction are homogeneous with regard to number of combining sites and dissociation constants of the combination at these sites Now death rates and behaviour with simple haemolysins indicate that bacteria and red blood corpuscles are not homogeneous. It is only with purified antigens, such as crystalline egg albumin, that homogeneity can be assumed It is desirable that such antigens should be used in all work on theoretical aspects of the subject

It is possible to measure the amounts of antibody or antigen free and combined, even when a precipitate is not formed and a particulate antigen cannot be centrifuged off, if it can be assumed that the antigen-antibody compound is highly irreversible Using this method Biltz (1910) found that the curve of neutralization of tetanolysin by antitoxin fitted the Freundlich isotherm, but would also fit an equation based on the Law of Mass Action Burnet (1931, a) found that the neutralization of staphylococcal toxin agreed with the supposition that the toxin combined stoichiometrically with antitoxins and that further toxin was adsorbed on the compound according to Freundlich's equation On the other hand Healey and Pinfield (1935) concluded that diphtheria toxin and antitoxin combine according to the laws of multiple proportions forming two compounds, one of which contains twice as much antitoxin as the other

Some importance has been attached to the conformance of some of these data with Freundlich's isotherm The data of Eisenberg and Volk and some of those of Cromwell fit very well Cromwell suggests that the deviations found with some sera may be due to the "haemolysinoids" demonstrated by him The results of Dreyer and Douglas do not fit this equation

It will be realized from Chapter I that the conformity or nonconformity of the combination of antibody and antigen with the Freundlich isotherm sheds no light on the question whether this combination is to be regarded as a chemical combination or an adsorption These experiments, however, suggest an interesting question, how, on any theory, can the particulate antigens accommodate amounts of antibody so many times greater than the amount required to affect the stability of their suspensions or their cell membranes? It is probable that these large quantities are only loosely combined, for de Kruif and Northrop (1922–3, b) found that only twelve minimal agglutinating doses would combine with typhoid bacilli sufficiently firmly to withstand washing

P The Titration of Antibodies and Antigens

The zone phenomenon has an important bearing on the titration of antibodies and antigens The procedures usually adopted are based

on the limiting concentrations which will agglutinate or give visible precipitates, that is, upon the position and slope of the right-hand boundary *BD* (Fig XXIII), or possibly of the left-hand boundary *BC*. Now these two boundaries vary considerably in their slope in different reactions and are particularly liable to be affected by non-specific factors such as the amounts of serum proteins. If α procedure is used for estimating the amount of antibody in serum, the limiting dilution of antigen will be inversely proportional to the dilution of the serum when the boundary is in the position *BD'*, this was found with some antipneumococcal sera by Sobotka and Friedlander (1928). But when the line is in the position *BD"*, as found, for example, by de Kruif and Northrop (1922–3, a), the results will be opposite, the weaker the serum the higher will be the limiting dilution of antigen with which it will give precipitation or agglutination. Cromwell (1925) found that, when simple protein antigens were used, the boundary was vertical, the limiting dilution of antigen which gave a precipitate was independent of the degree of dilution of the serum. In agreement with this, Culbertson (1932) found little difference in the limiting dilutions of egg albumin which gave precipitates with a large range of antisera. Morgan's (1924) results using Type II specific polysaccharide were similar to those of Cromwell. Hooker and Boyd (1932) removed varying proportions of antibody from an antiserum to atoxyl-azo-casein by precipitation with antigen, again the limiting dilution of antigen required to form a precipitate was the same for the various supernatant fluids obtained. Culbertson (1932) and Satoh (1933) found no relation between estimates of antibody by this method and by the antibody dilution or by the amount of antibody protein precipitable. The use of the end point in the α procedure for the titration of antibodies is therefore unsound, although it may be applicable in certain cases. There appear to be no objections to its use for the estimation of simple antigens, in which there is no likelihood of variations in the slope of *BD*.

When β procedure is used (that is, when the columns are followed) for estimating the strength of an antiserum, the results again will depend on the slope of *BD*, or, with high antigen concentrations, *BC*. These slopes may differ with different sera, as Sobotka and Friedlander found. So that two sera might be found to be of equal strength when titrated against one concentration of antigen but to differ when another concentration of antigen was used. In sera of which the antibody has been largely destroyed or removed, the proportion of non-specific protein to antibody has been raised considerably. These non-specific factors affect the limiting dilutions which will agglutinate or give precipitates, and titrations of such sera should be regarded with suspicion.

The optimum proportions method of Dean and Webb (1926) does not seem open to the objections raised against the usual methods, as it does not depend on the width of the zone. It has been used successfully by Taylor, Adair, and Adair (1932) for the estimation of protein antigens. Smith (1932) found that with unconcentrated antisera to

pneumococcus Type I the results, using this method, with the specific polysaccharide as antigen, agreed with mouse protection tests Also Culbertson (1932) found that determination of the neutral[1] point (which usually agrees with the optimum proportions) was a satisfactory method of titrating antisera to egg albumin , it agreed roughly with estimations by antibody dilution (β procedure) but not at all with those by antigen dilution (α procedure) The difficulty with the optimum proportions and neutrality methods is that little difference may be detectable over a wide range

Hooker and Boyd (1935) have proposed a method of estimating very small quantities of antigens, based on the fact that, in the region of antibody excess, the rate of flocculation is proportional to the concentration of antigen

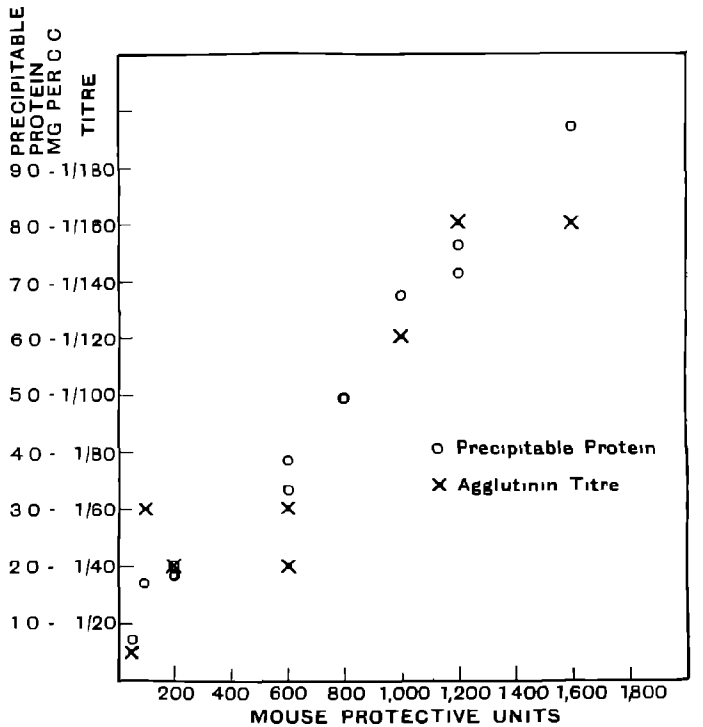


FIG XXVII Relation between mouse protection units, protein precipitable by the specific polysaccharide, and agglutinin titre in antisera to Type I pneumococcus (Heidelberger, Sia and Kendall, 1930)

If it is accepted that the protein, derived from the antiserum, which is found in a specific precipitate is the actual antibody, the

[1] That is the point at which both antigen and antibody, or neither antigen nor antibody, are present in the supernatant fluid after precipitation

obvious method of measuring antibodies is to estimate the maximum protein precipitable by the appropriate antigen This method was applied by Heidelberger, Sia and Kendall (1930) They found the maximum protein precipitated by S S S I ran closely parallel to the mouse protection units in Type I antipneumococcal sera (Fig XXVII) The method has also been used by Culbertson (1932) with egg albumin as antigen He found it inapplicable when the antigen was a mixed one, such as horse serum If the maximum precipitation occurs near the optimum proportions and the ratio of antigen to antibody globulin in the precipitate remains constant, this method will give approximately the same results as the optimum proportions method Since Marrack and Smith (1930) found that the amount of precipitate per unit of diphtheria antitoxin is approximately constant, the results by this method would agree with the Ramon method However, the ratio of antibody protein to antigen at the point of maximum protein precipitation may vary by as much as 30 per cent either way The antibody protein precipitated is not therefore an exact measure of the antigen combining power of an antibody solution A more exact method has been suggested by Heidelberger and Kendall (1935, b) Since the equation (3) in Section N of this chapter, is that of a straight line, an antibody solution can be characterized by two points on this line. This can be done by estimating the amounts of protein precipitated from a given volume of antibody solution by two different amounts of antigen From these estimations it is possible to calculate A, which in the case of Type III antipneumococcal sera is the amount of antibody protein precipitated at the beginning of the neutrality zone , and R, the ratio of antibody protein to antigen at this point

A series of estimations may be made of the amount of protein in the precipitate formed by varying amounts of antigen when added to 1 c c of any particular serum A curve can then be plotted with the amounts of protein precipitated as ordinates and amounts of antigen as abscissae It is then possible to estimate the amount of antigen in a solution by adding an appropriate amount of this antigen solution to the 1 c c of the antiserum and estimating the amount of protein in the precipitate formed The amount of antigen corresponding to this amount of protein can then be read off from the curve This method has been used by Heidelberger and Kendall (1932, b) for estimating small amounts of specific polysaccharides, and by Goettsch and Kendall (1935) for estimating albumin and globulin in body fluids

Q Immunization

Although substances other than proteins, polysaccharides, for example, may give good reactions with antisera, protein has usually been found necessary for immunization For example, antisera are produced by injecting lipins mixed with serum (Landsteiner and Simms, 1923) However, Gonzalez and Armangué (1931) succeeded in immunizing rabbits with the Forssman antigen adsorbed on kaolin Landsteiner and Jacobs (1932) confirmed this, and also

prepared a haemolysin by immunizing with the antigen adsorbed on collodion particles Zozaya (1932) obtained agglutinating and precipitating sera of moderate strength by injecting rabbits and horses with various polysaccharides, so adsorbed. More recently Zozaya and Clark (1933) have found that protection against subsequent doses of pneumococci can be obtained by the injection of very small doses of pneumococcal polysaccharides They considered it essential that the doses should be small and that surplus polysaccharide should be washed off collodion particles if these were used as adsorbents Landsteiner and Jacobs (1932) failed to confirm Zozaya's (1932) paper

The polysaccharides used in the earlier experiments had been considerably changed in the process of purification For example, the Type I pneumococcal polysaccharide had lost an acetyl group, and Avery and Goebel (1933) found that mice, though not rabbits, could be immunized by injections of the natural acetylated polysaccharide Heidelberger, Kendall and Scherp (1936) have shown that all the pneumococcal polysaccharides are considerably degraded by the earlier methods of preparation Numerous workers have shown that mice could be immunized to pneumococci by injections of non-protein preparations of pneumococci The immunity displayed in these instances is protection against virulent bacteria , however, demonstrable humoral antibodies have been also produced by non-protein antigens For example, a product from *Bact aertrycke* which contained no demonstrable protein produced agglutinins and precipitins when injected into rabbits (Raistrick and Topley, 1934)

There is, however, a striking, though not absolute, difference in the immunizing power of protein and non-protein This is well illustrated by S S S III and S S S III coupled to protein (Avery and Goebel, 1931) Injections of the protein compound not only immunize rabbits to pneumococci, but produce sera which will confer passive immunity on rabbits, besides agglutinating pneumococci and precipitating S S S III

The failure of gelatin to act as a full antigen has been attributed to the absence of aromatic groups Hopkins and Wormall (1933, b) found that attachment of aromatic groups by the action of phenyl-*iso*-cyanate did not convert gelatin into a full antigen , also Medveczky and Uhrovitz (1931) state that benzoylated gelatin is not antigenic However, Adant (1930) obtained a serum from rabbits immunized with gelatin-azo-atoxyl which gave precipitates with this compound ; and Hooker and Boyd (1933), using the same antigen, obtained a serum which would give precipitates with atoxyl-azo-casein and atoxyl-azo-egg-albumin, though not with the gelatin compound It is possible that the failure of gelatin to produce antibodies may be due to its simple structure or physical properties, not merely to the absence of tyrosine Other proteins which lack certain amino-acids (edestin and gliadin which lack lysine, zein which lacks tryptophan and lysine) will act as full antigens

Although immunization with the break-down products of protein is usually unsuccessful, Landsteiner (1919) obtained precipitins by immunization with large doses of peptone Landsteiner and van der Scheer (1930, 1931, b) also obtained precipitins by injection of azo-compounds of proto- and heteroalbumose

As an hypothesis of the way in which antigen used for immunization stamps on a large number of antibody molecules a specific conformation, Breinl and Haurowitz (1930) suggested that in the synthesis of serum globulin the arrangement of amino-acids is modified by the polar forces of the antigen which has been retained in the cells where serum globulin is synthesized This idea is amplified by Mudd (1932). He considers that the antigen may be regarded as a synthesizing enzyme Amino-acids (or other building stones out of which antibody may be synthesized), which conform to the distribution of polar forces on the antigen surface, are attracted to that surface, and the composition and spatial arrangement of the protein or other complex structure synthesized is determined thereby In order that the antigen should play such a part in the synthesis some degree of fixation would be necessary, and this may be the reason why some " carrier " protein or adsorbing particle is necessary

This theory is in good accord with a suggestion of Carothers (1935) that orientation of amino-acid molecules necessary for protein synthesis, *in vivo*, takes place at surfaces under the influence of the fields of force of those surfaces This adaptation to antigen would take place during the synthesis of the peptide chains If as suggested by Wrinch (1937), proteins are built up of layers, the antibody will not lose its adaptation to antigen when it assumes the globular form

A difficulty that arises with all theories of antibody production is the prolonged formation of antibodies after the injection of antigen and particularly the phenomenon of " anamnesis "—the stimulation of a fresh production to one antigen, when a new antigen is injected It seems necessary to suppose that immunization has some permanent effect on the organism The above theory meets this difficulty as well as does any other , it involves the supposition that the antigen must be a substance which can enter and remain in those cells of the immunized animal in which antibodies are synthesized

According to a theory which may be described as the mirror image to that put forward above, antibodies are formed from altered antigens This theory is still less able to explain anamnesis, and is disproved by quantitative considerations Heidelberger and Kendall (1930) showed that the weight of antibody globulin produced was at least five times the weight of antigen injected Hooker and Boyd (1931), basing their argument on the data of Topley (1930), have shown that the theory involves serious difficulties In a later paper (1932) they consider the question from the point of view of the precipitin reactions obtained with azo-proteins The general line of their argument may be put briefly as follows If the active part of the antibody is manufactured from the antigen, each combining site of the antibody must contain a determinant group of the

antigen in a form sufficiently unaltered to confer specificity In the case of the antigens made by coupling atoxyl to proteins an arsenic atom is an essential part of the determinant group, one must, therefore, be present in each combining site of the antibody From considerations of the proportion of antigen to antibody at optimum proportions, and of the minimum numbers of determinant groups per molecule of antigen necessary to produce precipitation, they conclude that 1 c c of antiserum must contain so many combining sites that its minimum content of arsenic, contained in these sites, must be $1\ 7 \times 10^{-7}$ g of arsenic Now Berger and Erlenmeyer (1931) found that such an antiserum contained less than 3×10^{-10}g of arsenic per c c, from which it follows that the essential specific part of the antigen cannot be built into each combining site of the antibody Hooker and Boyd also advance another line of argument, based on assumptions as to molecular weights of antibody and antigen, which demands an even larger arsenic concentration in the antiserum This argument, however, is less convincing as it cannot be assumed that the molecular weight of casein is not altered by the rather violent treatment it receives in coupling to atoxyl The first argument does not even involve the assumption that antibody is a protein

As immunization is prolonged the characteristics of the antibody change It becomes less specific, a change which has been attributed by Burnet (1934) to the appearance of binding sites for minor antigenic groups In one example studied by Heidelberger and Kendall (1935, c) the constants R and A in the equation (3) of section N both rose But the figures in the paper of Taylor, Adair and Adair (1934) suggest that the increase of R is not invariable Joffe (1935) found that, when immunization was prolonged, the isoelectric points of bacteria fully sensitized with the 'rough' agglutinin so obtained shifted from above pH 5 2 towards the alkaline side (in the extreme case to pH 5 9) Similar effects were not found with the 'smooth' agglutinin The H agglutinins also became resistant to digestion by pepsin and trypsin (Rosenheim, 1937) Rosenheim has suggested that this is due to the appearance of amino acid groups, adapted to bind antigen, buried in the peptide chains and only available to function as binding groups when uncovered by the action of the enzymes The multiplication of such groups might be associated with chemical changes sufficient to cause a change of isoelectric point

If the claims of Jermoljewa and Bujanowskaya (1930) to have obtained precipitins by the injection of simple amines are confirmed, a complete revision of ideas on the formation of antibodies will be necessary

R The Unitarian Hypothesis

In order to avoid misunderstanding it is as well to quote Zinsser's (1931) definition of the unitarian hypothesis " By such a conception of antibodies we do not imply that a complex cell like, for instance, a typhoid bacillus can only give rise to one variety of antibody only.

There may be found a specific sensitizing antibody against the major chemical constituents, and other sensitizers against the other antigenic substances enclosed in the same cell body or contained in the same antigenic solution But we do mean that, were we working with a single antigen in a pure state, one variety of antibody would be produced This would be present in the form of a serum constituent specifically capable of uniting with the antigen As a result of the union the antigen is altered in its physical and perhaps to some extent in its chemical behaviour The resultant reactions which may be observed with this sensitized antigen (agglutination, precipitation, bactericidal phenomena, opsonization, or sensitizing effect on the anaphylactic sense) would be determined, not by differences in the nature of the antibodies with which the antigen had united, but rather in the co-operating substances (alexin, leukocytes, tissue-cells) and by the environmental conditions under which the observations are made "

Dean (1916) based a similar hypothesis on his conception of the fundamental mechanism of immunity reactions—the aggregation of globulin particles round the antigen A similar point of view has also been maintained by Eagle (1930) Since the conception of this mechanism outlined above may be considered an amplification of that of Dean, it is equally in accord with the unitarian hypothesis

The question then arises whether any one process could produce all the various reactions encountered It is not difficult to imagine that the very similar precipitin and agglutinin reactions may be brought about by the same primary process , the demonstration by Jones (1927, b, 1928, b) that a precipitin for a given protein will agglutinate collodion particles coated with this protein, show that this is actually the case It might be objected that the collodion particles were carried down in a precipitate of antibody and egg albumin which had been dissociated off But Landsteiner and van der Scheer (1936) found that stromata of red blood corpuscles, to which a determinant group had been coupled by an azo link, were agglutinated by antisera to proteins containing this determinant group In this case there is no question of the dissociation of soluble protein from the insoluble particle Work by Mudd and colleagues (1930), with collodion particles on which protein had been adsorbed, shows that opsonization can also be brought about by the same antibody that forms precipitates and agglutinates The precipitin for Type III pneumococcal polysaccharides, obtained by Avery and Goebel (1931) by immunizing with this polysaccharide combined with a protein, will also agglutinate Type III pneumococci and protect mice specifically against Type III infection The demonstration by Reiner and his co-workers (1929) and Freund (1929) that a single non-specific reagent—tannin—will bring about several of the effects of antibodies (agglutination, ability to adsorb and be lysed by complement, and readiness for phagocytosis) also shows that it is not necessary to postulate more than one agent to account for diverse immunological reactions

Antisera to the pneumococci are particularly suitable for testing the unity of antibodies, since they give numerous reactions and it is possible to obtain one antigenic component of the pneumococci—the polysaccharide—practically pure Protective antibody is precipitated by the specific polysaccharide (Felton and Bailey, 1926, b), and can be recovered from the precipitate (Types I and II, Felton, 1932) Purified antibody (Type I) recovered from the precipitate formed with S S S I will also agglutinate pneumococci , and, when the original antiserum was rabbit serum, will fix complement in presence of S S S I, and will sensitize guinea-pigs to S S S I (Chow and Wu, 1937, b) The opsonic effect is inhibited specifically by the corresponding polysaccharide (Ward and Enders, 1933) The protective, precipitating, agglutinating, complement-fixing and opsonic titres of antipneumococcal sera (especially to Type I) run parallel in sera and in purified preparations (Felton and Bailey, 1926, a , Felton 1931)

There is some dispute whether antisera can be separated into fractions manifesting different specific effects Otto and Shirawaka (1924) claimed to separate precipitin and anaphylactic antibody by electrodialysis, but this was not confirmed by Doerr and Hallauer (1927), who also could not demonstrate any separation by ammonium sulphate precipitation (1926) Strumia (1930) and Mudd (1930), with their colleagues, found that various activities of antisera to acid-fast bacilli and of anti-protein sera ran parallel in the serum fractions they prepared It is possible that one function of an antiserum may be destroyed while another is left intact, but this does not imply that two antibodies are involved Thus diphtheria antitoxin will still neutralize toxin after extraction with alcohol and ether (Hartley, 1925, b), although it no longer gives a precipitate with toxin , moderately heated sera may combine with bacteria but not agglutinate them (Shibley, 1929 , Jones, 1927, a) , in these cases it is probable that only one antibody is involved Eagle, Smith and Vickers (1936) have stressed the great difference in the rates of inactivation of the different antibody functions of sera when treated with diazo-benzene-sulphonic acid They suggest that this is evidence of the presence of distinct antibodies which differ in the number and type of specifically reacting groups However, agglutination occurs in dilutions of antiserum which will not form precipitates with molecularly dispersed antigen It may be supposed that antibody molecules that have too few combining sites to form precipitates with molecularly dispersed antigens may agglutinate bacteria Eagle, Smith and Vickers considered that Type I antipneumococcal serum, treated with the diazo compound, which will agglutinate pneumococci but not form a precipitate with S S S I, did not combine with S S S I If this were the case it would leave important theoretical consequences They based their conclusion on the fact that mixtures of S S S I and treated serum, in which no precipitate is formed, form a precipitate when untreated serum is added This is, however, what would be expected if the treated antibody formed a relatively dissociable compound with S S S I from which it was displaced by untreated

antibody This would be comparable to the displacement of toxoid from its combination with antibody by toxin (Heidelberger, 1930).

Dean (1916) pointed out that the demonstration of complement-fixation by a mixture of antigen and antibody without precipitation, or vice versa, does not imply that the two reactions are brought about by different antibodies The proportion of antigen to antibody most suitable for complement fixation is not the same as the optimum for the production of a precipitate Nor in view of what has been said about the titration of antibodies can much importance be attached to discrepancies between the titres of sera for the various reactions which they produce

There is no doubt that, as Zinsser suggests, a complex antigen, such as typhoid bacillus, can give rise to more than one distinct antibody For example, the antibodies to the flagellar and somatic antigens of the hog cholera bacillus can be separated by specific absorption It seems possible that different parts of an antigen molecule even may give rise to different antibodies This point has been investigated by Heidelberger and Kendall (1934) using as antigen the azo-protein, *R*-salt-azo-benzidine-azo-egg-albumin The antigen appeared to contain no unchanged egg albumin, as it only exceptionally reacted with anti-egg-albumin sera Antisera to this compound formed a precipitate with egg albumin Egg albumin, if added in sufficient amount, precipitated all the antibody to the azo-egg-albumin compound from one of the sera studied From others it precipitated a large proportion of this antibody On the other hand, the azo-egg-albumin precipitated almost all the antibody that would react with egg albumin It therefore appeared that this single molecular species used as antigen would give rise to some antibody molecules that would react only with the azo-compound and some that would react only with egg albumin , but that most of the antibody molecules formed would react both with egg albumin and the azo compound This Heidelberger and Kendall attributed to the presence in the antigen molecule of major antigenic groups A (in this case the *R*-salt-azo-benzidine groups) and minor antigenic groups B (in this case groups common to the azo-egg-albumin and unchanged egg albumin) Most of the antibody molecules formed carried receptor sites to both A and B, a few to A only, and still fewer to B only The behaviour of such an antiserum is wholly different from that of one produced by immunizing with two wholly distinct antigens such as egg albumin and serum albumin (Dean, Taylor and Adair, 1935) Two such distinct antigens, A_1 and A_2, produce two distinct sets of antibody molecules a_1 and a_2 , antigen A_1 will precipitate only a_1 and leave the amount of A_2 unaltered

When more than one antibody is present in a serum the question may arise whether both can be carried on the same globulin molecule Hektoen and Boor (1931) immunized a rabbit with 35 antigens , the serum reacted with all but 3 After absorption with one of the antigens the serum still reacted with most of the others The antihaemoglobin precipitins seemed as specific as those that develop

from the injection of a single haemoglobin In this case, if we suppose all the antibodies are separate and there is 1 mg of each antibody globulin per 1 c c (a moderate amount), the total amount of antibody globulin in the rabbit's serum was 32 mg per c c , which is not impossible

S Ehrlich's Side-Chain Theory

Ehrlich's side-chain theory, providing as it does a concrete picture of the processes involved, and correlating them with those of normal metabolism, must lie at the back of all speculation on immunity According to this theory, there are, attached to the protoplasm of animal tissues, innumerable side chains—the receptors These receptors are concerned, physiologically, with the absorption of food molecules, which also have side chains—the haptophore groups It is only when fixed by combination of haptophore with receptor that food molecules can be incorporated with the cell protoplasm These receptors also combine with haptophore groups of other foreign molecules, and may be formed in excess and detached from the cell protoplasm, forming antibodies Further reactions, after the specific combination of haptophore and receptor, are produced by other groups on the antigen and antibody

The essential features of the theory, as far as it is involved in this discussion,[1] may be said to be (1) combination of antigen and antibody is a process distinct from any subsequent reaction, such as agglutination or precipitation , (2) specific combination takes place between certain side chains of antigen and antibody , (3) subsequent reactions are produced by groups other than the combining groups

The theory is rendered more plausible by the fact that it fits many of the phenomena of enzyme actions In these reactions two stages can be recognized (1) combination between certain groups of enzyme and substrate, and (2) some secondary stage, such as hydrolysis of the substrate This second stage, however, is not attributed to special groups of enzyme or substrate, but may be due to some special feature of the enzyme, such as the distance between two combining groups (Haldane, 1930)

In immunity reactions the distinction between the two stages, combination and subsequent reaction, was demonstrated by the action of modified antibodies, such as agglutinoids, which combine with bacteria but do not agglutinate them This feature and the second feature—the dependence of the combination of antigen and antibody on specific side chains—are much more obvious in the reactions obtained with artificial antigens made by attaching determinant groups to proteins In these cases the combination of the side chain (the " determinant group ") with the antibody can be demonstrated directly In many cases this combination is not followed by any further reaction, so that the further reaction cannot be attributed to the combination alone However, as with enzyme

[1] It is not necessary to discuss here other features connected with complement fixation and with the action of toxins

reactions, the further reaction, when it occurs, is probably not due to some other definite group in the antigen or antibody, but to the general conformation of the molecules Whether the binding sites of natural proteins can also be regarded as side chains, or are formed by the general configuration of the surface of the moleucle, is uncertain The experiments of Landsteiner and van der Scheer (1932) on polypeptides suggest that certain groups of amino acids may act as side chains

In Ehrlich's theory antibodies are supposed to be receptors for normally occurring foodstuffs, formed in excess and broken off It is hardly conceivable that so many diverse normal receptors could exist and this part of the theory is better replaced by that advanced by Breinl and Haurowitz (1930) and by Mudd (1932)

An attractive aspect of the theory is the suggestion that other physiological processes, besides enzyme actions, may depend on a specific combination similar to that found in immunity reactions It is conceivable that the direction of many metabolic processes may depend on such a combination, which either anchors some substance in a cell or removes it from the field of possible activity Immunity reactions, with artificial antigens, afford a unique means of investigating the possibilities of such specific combination

It is true that the union between antigen and antibody must in many cases be classed as an adsorption The insistence of Bordet on this aspect of the reaction may have supplied a valuable stimulus to the study of such questions as the influence of salt concentration But even here, in so far as he considered that specific combination depends on a specific configuration of atoms representable by chemical formulae, the developments of immunology appear to have confirmed Ehrlich's views

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